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Genetic Aspects of Bacterial Endospore Formation

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INTRODUCTION

Bacteria of several genera are able to form endospores when subjected to certain starvation conditions. The endospores are dormant forms; they have structural and biochemical characteristics that distinguish them clearly from the corresponding growing organisms. Spore formation is looked on as a primitive form of cellular differentiation, since it has several features in common with cellular development in higher organisms. Experimentally, the process has the advantage that up to 90% of a population of cells can be induced to form spores in a relatively short time in a defined medium. The population can be induced to sporulate fairly synchronously, although each cell in a population appears to need no interaction with its neighbors—there is no cell-to-cell interaction to form a complex multicellular structure.

The literature on sporulation is voluminous. Most of the work has been with members of the genera *Bacillus* and *Clostridium*, and there are now several general reviews of the subject (20, 57, 87, 122, 166, 196, 197, 197a, 215, 267, 293). This review is limited to genetic aspects, and

consequently the article emphasizes work with *Bacillus subtilis*, as systems for genetic exchange have only been extensively exploited with this species. It is widely assumed that many of the features of sporulation are common to many, or all, species of endospore former, and we have included discussion of species other than *B. subtilis*, usually other *Bacillus* species, where this seemed appropriate. However, we have not attempted a comprehensive review of the validity, or generality, of this assumption. Nor have we attempted to explore in detail the extent to which bacterial endospore formation is a valid and useful model for cell development in higher organisms. Much of the earlier work on the genetics of spore formation has been well covered in two older reviews (20, 267), whereas Hoch has provided a good succinct summary of the subject (134). Consequently, we concentrate on the more recent literature. We begin with an outline of the sequence of morphological and biochemical changes during sporulation. This is followed by a brief characterization of the mutants that are unable to sporulate. These sections provide a framework of basic information on which we then attempt to build a picture of the way in which the initiation and subsequent events of sporulation are controlled. In an appendix, the different sporulation loci of *B. subtilis* 168 are characterized in detail.

MORPHOLOGICAL AND BIOCHEMICAL CHANGES DURING SPORE FORMATION

In liquid media, sporulation is usually triggered by starvation for a carbon or nitrogen source, and sometimes by phosphate starvation. Two methods are generally used. First, an exhaustion procedure, whereby bacteria grow in the medium, use up some essential nutrient, and then sporulate. The efficiency of sporulation and the degree of synchrony obtained depend on the medium used. Second, a replacement technique whereby bacteria that are growing exponentially in a rich medium are transferred to a poor medium. The replacement method gives a more clearly defined starting point and, generally, a better synchrony throughout the process. It is more suitable for certain experiments (such as radioactive labeling), since the replacement medium is a chemically defined medium. The exhaustion procedure is more convenient for large-scale experiments and is, of course, more comparable to the process as it occurs on solid media. For most purposes the two procedures yield the same results, although this may not be the case when the composition of the medium is important (167, 235, 322). With *B. subtilis* at 37°C, heat-resistant spores appear some 7 to 8 h after the point of induction.

The sequence of morphological changes resulting in the formation of a spore has been elucidated by electron microscopy and is similar for all species of *Bacillus* and *Clostridium* that have been examined (reviewed in [82]). Although the process is continuous, it is convenient to divide it into the stages defined by Ryter (253) and represented by Roman numerals (Fig. 1). Growing (vegetative) bacteria are designated as stage 0. Stage I was originally identified as condensation of the two nuclei of the vegetative cell to form a single axial filament of chromatin, but this has been challenged (20, 199, 329, and see below). Stage II designates completion of a septum formed by membrane invagination and growth at one pole of the cell. Yamamoto and Balassa (329) have identified wall protrusions, or spikes, at the site of membrane invaginations. They suggested that formation of these spikes before septation should be designated stage I, but this designation is not used here. There is little wall material visible in micrographs of the spore septum itself, and it is more readily visualized with some species than with others (140, 248, 329). Freese (87) has suggested that at least some peptidoglycan synthesis is necessary at this time to give direction to the membrane synthesis and so ensure that a septum is formed. The peptidoglycan that is synthesized during septation is then apparently digested away (115) so that the bacterium can proceed to stage III, which is the formation of a protoplast free within the mother cell. This is brought about by bulging of the spore septum into the cytoplasm, followed by movement of the points of attachment of the ends of the septum to the pole of the mother cell. Stage IV is the deposition of primordial germ cell wall and cortex between the membranes of the spore protoplast. Deposition of spore coat around the cortex defines stage V, and stage VI is the "maturation" of the spore, at which time it develops its characteristic resistant properties. During stage VII (not shown in Fig. 1), the mother cell lyses and releases the completed spore. Detailed discussion of the morphological changes is given elsewhere (58, 82, 159, 215, 253).

The biochemical activities that appear during sporulation may, or may not, be necessary and specific for the process. They can be divided into several functional classes in an attempt to categorize this matter (87, 122, 196). (i) Appearance of components that do not occur during growth and are found only in sporulating cells and that are apparently necessary for spore formation. Examples might be the peptidogly-

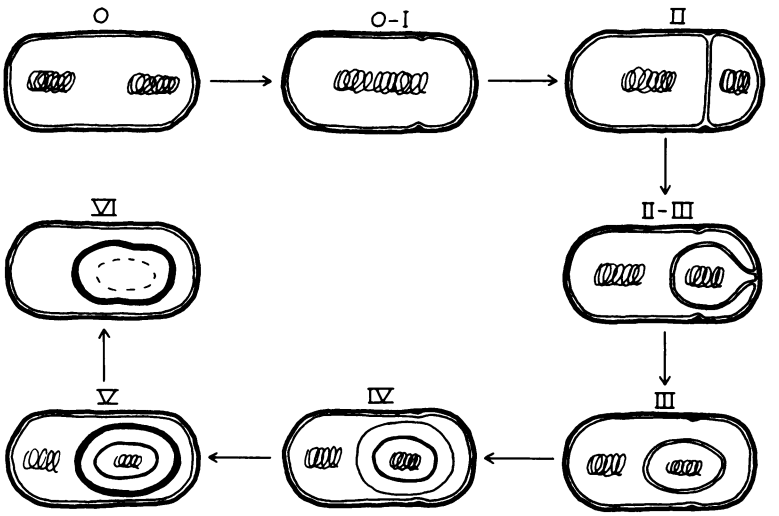


FIG. 1. *Schematic representation of the morphological changes associated with the stages of sporulation (Roman numerals).*

can of the cortex (which differs structurally from that of the cell wall), and the proteins of the spore coat. (ii) Changes in vegetative functions that are necessary for sporulation. An example would be the increase in activity of the enzymes of the tricarboxylic acid cycle, which is necessary to maintain adequate adenosine 5'-triphosphate (ATP) levels under the starvation conditions that promote sporulation (87, 122). (iii) Side effects triggered by the primary sequence of events during sporulation, but which do not themselves play a role in the process. Unless a suitable mutant happened to have been studied it would be difficult to distinguish between changes of this type and those of type (i). The increase in metalloprotease during the early stages of sporulation (56, 117) may fall into category (iii). (iv) Changes that result from the nutritional shift-down used to initiate sporulation but that are unconnected with sporulation. Examples are the increase in α-amylase and arginase activities (171, 267). (v) Appearance of components that may be required during subsequent germination of the completed spore but which have no other role in spore formation. A failure to germinate indicates a defective (or dead) spore, and therefore a defect in spore formation. Nevertheless, it is convenient to consider these components as a separate category. An example here may be the formation of alanine dehydrogenase (92, 321).

The study of mutants has been useful in attempting to place an event into one of the above categories. Mutations in unnecessary events should not affect sporulation. Mutations in necessary events should affect sporulation, and, if the event is sporulation specific, should not affect vegetative growth. Conversely, biochemical events associated with sporulation may not be expressed in mutants unable to sporulate. In practice, such mutants are readily isolated. On a variety of solid media, colonies of the wild type (Spo^+) produce a brown pigment, whereas colonies of asporogenous (Spo^-) mutants are poorly pigmented (147, 269). This has provided a convenient method for isolating a large number of asporogenous mutants of *B. subtilis* which may be blocked at any one of a variety of stages of sporulation. For other species, in which the pigment is not formed, colonies of asporogenous mutants may still be distinguishable from those of the wild type by their morphology (267). Colonies of *B. subtilis* mutants blocked late in spore formation are less readily distinguishable from the wild type than those of early blocked mutants (213). Consequently, it is possible that the method is biased against late-blocked mutants. However, Balassa has reported that in a large screening of colonies with the same pigmentation as the wild type no asporogenous mutants were detected (20). In an attempt to circumvent this difficulty, Millet and Ryter used replica plating to screen for chloroform-sensitive mutants (213), although the more convenient pigment identification of colonies of mutants remains the method of choice.

Many enzyme activities increase during sporulation (166), but we shall limit discussion here to those enzymes and biochemical changes that have been used as markers to classify sporulation mutants whose lesions have been mapped.

Protease. Exoprotease activity produced by *B. subtilis* soon after the initiation of sporulation has been shown to involve two major activities: a serine protease with an alkaline optimum pH and a metalloprotease with a neutral optimum pH (210, 232). The metalloprotease activity is unnecessary for sporulation, since mutants lacking its activity sporulate normally (117, 206). It is nevertheless associated with the process as a side product (56). Several lines of evidence indicate that formation of a serine protease is intimately involved in spore formation. All mutants of *B. subtilis* that lack this activity are unable to sporulate (267). Phenylmethylsulfonyl fluoride does not inhibit vegetative growth but blocks sporulation (55). As phenylmethylsulfonyl fluoride specifically inhibits the serine protease, this is consistent with a requirement for the serine protease. Similar results have been obtained with a released inhibitor, *m*-aminobenzeneboronic acid (99). For a mutant of *B. subtilis* that is temperature sensitive for sporulation, there is evidence that the serine protease has a reduced thermal stability (180, 181).

Mandelstam and Waites described a *B. subtilis* mutant with reduced exoprotease activity which showed reduced intracellular protein turnover, but was unimpaired in its ability to synthesize protein (201). This suggested that the exoenzyme did not play a direct role in sporulation, but rather its appearance reflected the presence of an intracellular enzyme that did play a direct role – possibly degrading some inhibitor of sporulation. More recent work with *B. subtilis* has indicated that there is more than one serine protease (212); it is not clear how these are related to each other.

Studies with other species serve as a warning not to rely solely on *B. subtilis*. Hypoprotease-producing mutants of *B. cereus* sporulate normally (3); *Bacillus megaterium* and *Clostridium pasteurianum* do not produce an extracellular serine protease (193, 211); *Bacillus brevis* produces little or no protease of any sort (277). Clearly, large quantities of extracellular serine protease are not specifically required for spore formation. Nevertheless, for the geneticist, production of extracellular serine protease by *B. subtilis* serves as a useful marker for *spo0* mutants.

Antibiotic. Antibiotic production is also one of the earliest events after the initiation of sporulation. Two antibiotics have been described for *B. subtilis*: bacilysin (243) and surfactin (30). Activity is usually determined against *Staphylococcus aureus* (271). Whether these antibiotics play an essential role in sporulation in this or other species, or are produced as side products, is at present open to speculation (62). Certainly their production is associated with the process, since many stage 0 mutants fail to produce them. Ray and Bose (237) have reported mutants of *B. subtilis* that are unable to produce an antifungal polypeptide antibiotic, mycobacillin, and are able to sporulate; it is not clear how this relates to the antibacterial antibiotics. Mutants unable to produce detectable antibiotic, but able to sporulate normally, have been reported for *B. licheniformis* (116), whereas *C. pasteurianum* wild type does not produce detectable antibiotic and yet sporulates normally (193). Thus, if these antibiotics have an essential role in sporulation, a few molecules, below the level of detection, must suffice.

Membrane changes. Several other characters have been used to distinguish stage 0 mutants from the wild type and later blocked mutants. These include sensitivity to the antibiotic of the wild type (112, 151), sensitivity to polymyxin (112), inducibility of nitrate reductase (33, 114), and sensitivity to certain bacteriophage (152, 153). All are thought to indicate alterations to membrane functions that are possibly important for spore formation. Several of these characters have been exploited to isolate partial revertants of stage 0 mutants (112, 113, 150, 151).

Energy production. Changes in the enzymes involved in energy production are known to occur at the start of sporulation. Foremost among these are increases in activity of enzymes of the tricarboxylic acid cycle (87, 120, 122). These changes are necessary for sporulation as mutants lacking an enzyme of the tricarboxylic cycle are blocked at stage 0 or I of sporulation (86, 300). Such mutants apparently cannot maintain the concentration of ATP necessary for sporulation (87, 220). Mutations for many enzymes of the tricarboxylic acid cycle have now been mapped (251). All cause a block at stage 0 to I of sporulation, although in certain circumstances this block may be overcome (220, 336). They are clearly not sporulation-specific mutations, since the lesions can affect growth under a variety of conditions. Warren has reported that a *spo0H* mutant (E22) did not show the increase in aconitase activity that was exhibited by the wild type under similar conditions (321). However, this activity has not been used extensively to characterise sporulation specific mutants.

Perhaps the earliest report of a change taking place in the activity of a metabolic enzyme during sporulation in the wild type and not in a

mutant was that of a particulate reduced nicotinamide adenine dinucleotide (NADH) oxidase (302). Although largely confined to *spo0A* mutants, the difference was not large (37) and this character has also not been used extensively.

Certain cytochromes are induced at an early stage of sporulation (80, 303). This induction is necessary for sporulation, as mutants lacking the cytochromes do not sporulate (303). The wild-type induction pattern is shown by *spo0E*, *spo0F*, and *spo0H*, but not by *spo0A* or *spo0B* mutants (303). The induction of menaquinone synthesis that occurs during sporulation is not seen in a *spo0H* mutant (322).

In an attempt to develop rapid methods to screen sporulation mutants for their respiratory activity, Balassa examined a wide range of tetrazolium compounds (19). Two of these, triphenyltetrazolium chloride and *p*-tolyltetrazolium red, could be used to distinguish certain mutants from the wild type. Unfortunately, the specificity of this test is not known.

Other events. The loss of ability to transcribe deoxyribonucleic acid (DNA) (189, 282) is now one of the most widely studied changes during sporulation. The change is not observed with stage 0, but is seen with stage II or later-blocked mutants (40, 282). However, the assay is difficult and has not been used extensively to characterize sporulation mutants.

Stage 0 mutants are often low in their competence to be transformed (267, 286). This property has been used to classify stage 0 mutants (203). It is a reliable marker when used under standard conditions in a particular laboratory, but may present difficulties when the results of different laboratories are compared.

During vegetative growth, alkaline phosphatase is repressed by inorganic phosphate (1). However, there is a 30- to 40-fold increase in specific activity during sporulation even in the presence of excess inorganic phosphate (102, 145, 321). The increase is associated with stage II (101). Under the same conditions, the enzyme is not formed by stage 0 or some stage II mutants, but is formed by all other sporulation mutants (49, 227, 320). Mutants have been described which have lost the ability to make the "vegetative" enzyme, but are unimpaired in their sporulation and produce the sporulation enzyme normally (107, 145). No mutants have been found with a lesion in the structural gene (107, 175), so that it is not possible to say whether the enzyme itself is necessary for sporulation. Mutants that are constitutive for its formation sporulate normally (209) so that premature synthesis of the enzyme does not interfere with spore formation. Phosphate starvation can trigger spore formation (111, 144), but it is not possible to deduce from this any role for alkaline phosphatase. It is worth noting that spore formation is not triggered by phosphate limitation in continuous culture (60) where alkaline phosphatase is also derepressed. This failure to sporulate could relate to the altered composition of the vegetative cell wall on phosphate limitation in continuous culture (73a).

Appearance of glucose dehydrogenase during sporulation has been associated with the transition from stage III to IV (49, 320). Mutants blocked at or before formation of the spore septum do not synthesize the enzyme. It is not known whether the enzyme has a specific role in sporulation, or merely reflects some other change during sporulation. The enzyme does have a role in germination (231).

Dipicolinic acid (DPA) is a compound unique to the bacterial endospore (216). Its synthesis occurs late in sporulation (317), and in mutants it is only formed by those that are blocked very late in the process (49, 82). Unlike the wild type, such mutants tend to lose DPA into the medium (49, 82). Mutants have been described that lack DPA, but form heat-resistant spores (121, 337). These do not retain their dormancy and are defective in germination, so that it is presumably for these purposes that DPA is required.

The capacity to synthesize spore coat proteins has also been used to classify sporulation mutants. Although deposition of coat material defines stage V, Wood showed by immunological means that the capacity to synthesize an alkali-soluble coat protein was present in mutants blocked as early as stage II (327). This and other results (5) suggested that the deposition of coat material at stage V resulted from the modification of preexisting precursors that had been synthesized very much earlier (reviewed in [6a]).

Sulfolactic acid is synthesized by sporulating cultures of *B. subtilis* (34). It is synthesized by mutants blocked at stage IV, but not by mutants blocked earlier (326). As it is not synthesized by other bacilli (34) its significance must be questionable. Presumably, it reflects some other change that is taking place at stage III to IV and which may be important for sporulation.

The most characteristic events of sporulation, development of refractility, of organic solvent resistance, and of heat resistance, have not been satisfactorily defined biochemically. However, they are among the easiest events to assay. The development of refractile (viewed by directly transmitted light), or phase-white

(viewed by phase contrast), prespores occurs at about stage IV when the cortex is being synthesized. (It should be noted that, although not strictly correct, many authors use refractile and phase-white interchangeably.) Mutants blocked at stages IV and V generally form feebly refractile (phase-grey) prespores. This stage is generally not stable in the mutants, although in some cases phase-grey cigar-shaped "sporlets" are released into the medium (49, 103, 320). After the development of refractility, the spore becomes resistant to a number of organic solvents, such as octanol and chloroform. Octanol resistance has been associated with the development of coat layers (stage V) (253). In keeping with this, all sporulation mutants are octanol sensitive, with the exception of a few that are blocked at stage V (24, 49). Milhaud and Balassa reported that *B. subtilis* develops resistance to different solvents at different times (208); this could provide a method for distinguishing different classes of late blocked mutants. All truly asporogenous mutants are, by definition, heat sensitive. However, heat resistance develops gradually in the wild type (208), and it may be possible to isolate very late-blocked mutants with a low level of heat resistance. The rather different case of oligosporogenous mutants that produce a low proportion of fully heat-resistant spores is discussed later.

CHARACTERIZATION OF ASPOROGENOUS MUTANTS

Genetic Mapping of Asporogenous Mutations of *B. subtilis* 168

Considerable information about sporulation can be obtained by examination of the morphological and biochemical properties of asporogenous mutants. This has been supplemented by genetic studies undertaken after the development of the techniques for genetic exchange in *B. subtilis* of transformation (284) and transduction (304, 305, 310). Mapping of asporogenous (*spo*) mutations has now reached a stage where it is possible to make a reasonable estimate of the number of genetic loci, and, more speculatively, the numbers of genes and operons, that are specifically involved in spore formation (see below, section on Nomenclature of *spo* loci, for the distinction made between these terms). This gives an idea of the complexity of the process. It also provides a basis for working out the ways in which the expression of loci is controlled. Bacteriophage PBSI-mediated transduction transfers about 5% of the donor chromosome (182) and is used to establish linkage on the genetic map by determining cotransduction frequencies of *spo* mutations with auxotrophic, or other, markers of known location. Generally, in transformation crosses, much less of the chromosome is integrated into the recipient genome and recombination frequencies are much higher. Consequently, transformation is more suitable for fine-structure mapping.

Historically, the first crosses involving *spo* mutations were by transformation (269, 270, 271, 285). These established that there were a number of *spo* loci. Later studies established that there were at least three, four, and five loci governing stages 0, II, and III, respectively (203, 250). The measure generally used for linkage by transformation between *spo* loci is the recombination index (RI) (170). In crosses with recipient *spo-1 aux*$^-$, and donors *spo-2 aux*$^+$ and *spo*$^+$ *aux*$^+$, where *aux* is an auxotrophic marker unrelated to sporulation, *spo*$^+$ and *aux*$^+$ transformants are scored independently. The RI is given by the formula RI = [*spo*$^+$/*aux*$^+$ (*spo-2* as donor)]/[*spo*$^+$/*aux*$^+$ (spo$^+$ as donor)]. If *spo-1* and *spo-2* are not linked by transformation, then the RI is 1.0. Values of 0.1 or less have been taken to indicate that the mutations are in contiguous genes or in the same gene (43, 195). In the subsequent discussion, mutations that are closely linked by transformation (RI $\leq$ 0.3) are generally considered to lie in the same locus (locus is not synonymous with gene, see below).

In most studies, the loci identified by *spo* mutations have been located on the chromosome by PBSI-mediated transduction (50, 137, 148, 227, 244, 306, 307). However, day-to-day differences in cotransduction frequencies mean that analysis by transduction alone may not give a precise map position, and so may not be adequate to assign mutations to specific loci (137, 148, 227). For any one transducing lysate, the differences are invariably seen as a loss of linkage with time. With some lysates, cotransduction frequencies have fallen from 90 to 20% within a matter of weeks, whereas, with other lysates, linkage values have remained constant for several years (P. J. Piggot, unpublished observations). Where such variation has been found, the initial (higher) values have given a more reliable indication of map distance in so far as this can be judged by other criteria.

Nomenclature of *spo* Loci

The system of nomenclature used here is an extension of the systems used to define sporulation loci in *B. subtilis* 168 by Ionesco et al. (148), Hoch and Mathews (136), and Young and Wilson (331). It also corresponds approximately to the system used by Piggot to enumerate sporulation operons (227). The loci are identified by

sporulation mutations. These are defined as mutations that affect spore formation, but do not affect vegetative growth under a variety of conditions. Thus, mutations are excluded that disrupt spore formation as a result of, for example, a lesion in the citric acid cycle. The definition is convenient, but need not indicate any fundamental distinction between *spo* and other loci. No distinction is made between oligosporogenous and asporogenous mutations (see section on oligosporogenous mutations). Mutations that are closely linked and which appear to disrupt the same function are considered to be members of a given locus (see below). Because of the inadequacy of the biochemical characterization of mutant phenotypes, it is generally not possible to say whether closely linked mutations lie in a single gene or in closely linked genes. Thus, locus is not synonymous with gene. Each locus may have its own control elements and so be considered an operon (as advocated by Piggot [227]), but this need not be the case.

Identification of distinct loci. When two mutations are sufficiently distinct genetically and/or phenotypically, they are considered to lie in different loci. The criteria used to make this distinction comprise the following. (i) Mutations that are separated by a "vegetative" marker on the genetic map are assumed to lie in distinct loci. (ii) Mutations that are unliked by transformation are assumed to lie in distinct loci. (iii) Mutations that cause blocks at different stages of spore formation (as defined by Ryter et al. [257]) are assumed to lie in distinct loci.

In certain instances, mutations that are (or may be) linked by transformation and give rise to clearly distinguishable blocks at the same stage have been placed in separate loci. This applies to the following pairs of loci: (i) *spoIVC* (no cortex)/*spoIVE* (almost complete cortex) (transformation crosses not performed), and (ii) *spoVD* (DPA^+ $cortex^+$ poor coat)/*spoVE* (DPA^- $cortex^-$ good coat) (weakly linked by transformation).

In other instances, mutations resulting in different phenotypes of the same stage have been placed in a single locus. In these cases, the individual mutations can apparently give rise to more than one phenotype so that the phenotypic distinction between mutations may be fortuitous. This applies to *spoIIA*, *spoIIE*, and *spoIIIA*. These various cases are discussed more fully in the descriptions of individual loci.

Naming of loci. *spo* is used for loci; Spo is reserved for phenotypes.

The designation includes the stage of blockage essentially as defined by Ryter et al. (257). However, no distinction is made between stages 0 and I; to avoid confusion with earlier literature, all mutations causing blocks at stages 0 or I are called *spo0* (see below). *spoII:* mutants have one or more sporulation septa, but no free prespore. *spoIII:* mutants have a prespore free within the mother cell; this prespore has no cortex, primordial germ cell wall, or coat. *spoIV:* mutants have prepspore with primordial germ cell wall and perhaps cortex, but no coat layers. *spoV:* mutants have coat layers around the prespore, and may or may not have complete cortex and/or primordial germ cell wall. An uppercase letter is used to distinguish different loci for the same stage.

Anomalies. Many mutations that have not been extensively characterized cannot be assigned to specific loci. This applies particularly to the "late" mutations described by Rogolsky (244) and Hoch and Spizizen (137).

In several instances *spo* mutations have been located in a particular region of the chromosome by transduction crosses, but have not been tested for linkage by transformation to other *spo* mutations of similar phenotype in the region. All are assumed to lie in a single locus, unless there is evidence to the contrary. The group of mutations for which there are the most complete mapping data is used to define the locus (for example, *spoIIA*); the other mutations assigned tentatively to the locus are designated with a (*c*) in Table 1 (*spoIIA*^{c}). Where there are known to be two *spo* loci for the same stage in a region, the mutations whose assignment is uncertain are (arbitrarily) placed in the locus that seems the more likely, e.g., *spo0A*^{c}; this placement is influenced by phenotype, relative frequency of occurrence of mutation in the two loci, and cotransduction frequency. Again, this is tentative, and is indicated by a (*c*).

We have not attempted to introduce a uniform system for numbering alleles within a locus. At present different laboratories use different systems, and strain isolation numbers are often used interchangeably with allele numbers. Previous suggestions of a uniform system (69, 331) have not come in to general use. This may be because there was no attempt to distinguish different loci. However, such a salutary example has discouraged us from attempting any similar exercise. We would only suggest that the information in Table 1 could provide a framework for the enumeration of alleles that have been shown unequivocally to lie in a particular locus.

Sporulation Loci in *B. subtilis* 168

The list of sporulation loci for *B. subtilis* include nine stage 0, seven stage II, five stage

TABLE 1. *spo loci*

Locus	Previous designation (if any)	Allele no. (or strain, isolation no.)	Linkage[a]		Order from multiple-factor crosses	References[b]
			Transformation	Transduction (%)		
spo0A	sp_{0A} *spo0A* *loc1* sp_{0C}	6U, 3NA, 5NA, 22NA 6NA, 7NA, 8NA, 10NA, 11NA, 14NA, 16NA, 106NA, 110NA 9V, 10V, 13V, 1Py	RI ≤ 0.17	15 *lys-1*	*spo lys trpC*	113, 148, 203, A
	spoA	A1-A14	RI ≤ 0.23	38–61 *lys-1*; 35–40 *trpC2*		37, 133, A
	group A	A102, 105, 107, 108, 109, 111, 115, 120, 124, 125, 126, 128, 129, 130, 132, 136, 149, 153, 156, 171, 173, 174, 185, 188, 190, 197, 204, 210, 211, 212	RI ≤ 0.1	34–80 *lys-1*; 46 *trpC2*		152, 153, A
		P10, Y13, NG6.21, 34(3F1)	RI ≤ 0.19	43–70 *lys-1*; 25–39 *trpC2*	*spo lys trpC*	50, 143, 227, A
spo0A[c]	*spoB*	B1, 2, 3, 5, 7, 8, 9, 10, 11		65 *lys-1*; 35–40 *trpC2*		137
		170-2		23 *tyrA*		306, 307
		P12		54 *lys-1*; 35 *trpC2*		50
		B36NG, B332H, BP97NG, BP3A	No recombination	58 *lys-1*; 35 *trpC2*		244
spo0G		14UL	Separate from *spo0A*	29 *lys-1*; 17 *trpC2*		148
spo0G[c]		*ts-5*		30 *aroD*		180
spo0D		8H		26 *phe-1*; 6 *ilvC1*		148
spo0B	*sp0B* *spo0B* *loc2*	3YB, 7U, 6Z, 6UA, 18U, 15U, 17NA, 9NA, 107H	RI ≤ 0.14 33–54 *phe-1*	50–98 *phe-1*		113, 148, 149
	spoB	83, 123, 125, 126, 136, 141, 149, 176	70–80 *phe-1*	>90 *phe-1*	*phe spo leu* *spo phe* *nic*	136
spo0B[c]	group B	42, 65, 68, 89, 91, 101, 106		90–100 *phe*		152,
		H_{12-14}		54 *phe*		306, 307
		98-8		20 *leu*		307
spo0F	*spoF*	111, 128, 129, 138, 167, 170, 187, 191, 197, 221	RI ≤ 0.2 75% *ctrA1*			136, A
	spoF	124	RI 0.1 to 0.5 with other *spo0F*			136
	spoB *loc1*	3U, 3Y, 23NA, 108C, 6V, 7V, 20UL	RI ≤ 0.1	14 *acf*		113, 148, 203, A
spo0H	*spoH*	H75, H81	RI ≤ 0.01 10–50% *cysA14*	>90 *cysA14*		136
		H12-3				307, A
		E22, NG7.17, 81, 84	RI ≤ 0.11 50–70% *cysA14*	95 *cysA14*	*cysA spo rif*	143, 227, A
spo0H[c]		C14-1		13 *cysA14*		307
		1		37 *ery*		306, 307
		B12, 13, 14		75–91 *str*		137
		B14NG, B4NA, B116NG, B37NA		79–96 *cysA14*; 80–91 *str*		244
	group C			Data not given		153
spo0J		87, 93	RI 0.39	48, 49 *cysA14*		143
spo0K		Z31	50% *trpS1*	42 *metC3*; 65 *argC4*		49, A
spo0E	*spoE*	E11		4 *ura-1*; 19 *metC3*		137

TABLE 1—*Continued*

Locus	Previous designation (if any)	Allele no. (or strain isolation no.)	Linkage[a]		Order from multiple-factor crosses	References[b]
			Transformation	Transduction (%)		
spo0E[c]	*spoE*	E161		2 *ura-1*; 27 *metC3*		137
		P2, P13, P19		79 *ura-1*; 23 *metC3*		49
		ts-4, ts-B8		Linked to *ura*		295
spoIIA	sp_{IIA}	26U	Linked to each other	≤80 lys-1; 45 *trpC2*	*spo lys trpC*	148, 149
	sp_{IIB}	4Z, 16U, 12U, 4SA	8–45% *lys-1*			
spoIIA[c]		E1, NG1.82, NG6.13, NG11.2, NG16.2, NG18.6	RI ≤ 0.21	≤94 *lys-1*; 70 *trpC2*	*spo lys trpC*	227
		P18		96 *lys-1*; 67 *trpC2*		49
		N2-2		36 *ser*		306, 307
spoIIB		Z3		90 *phe-12*; 54 *leu-8*		49
spoIIC		P9		43 *hisA1*; 58 *cysB3*		49
spoIID		NG17.22, NG17.29	No recombination with each other	12–19 *hisA1*; 10–15 *ctrA1*		52, 227, C
spoIIE		N25, NG2.6, NG9.3, NG14.22, NG15.4, NG17.15, NG20.12, U27, 95	RI ≤ 0.32 ≤64% *cysA14*	≤96 *cysA14*	*spo cysA rif*	143, 227
spoIIE[c]		P14		73 *cysA14*		49
		DG2		95 *cysA14*		52
		9Z		60 *ery-1*		148
		46-7 (NG)		25 *purA*		307
spoIIF		96		49 *metC3*; 21 *ura-1*		143, 334
spoIIF[c]	sp_{IIB}	20U, 72U		≤42 *ura-26*		148
spoIIG		E10, NG4.14, NG10.2, NG12.12, 279		≤83 *ura-1*; 5 *metC3*	*metC spoIIF spoIIG cysC7*	227, 334
spoIIIA		NG1.13, NG7.2, NG12.5, NG14.7, NG17.17, E34, 79, 86, 90	RI ≤ 0.51	≤54 *lys-1*' 44 *trpC2*	*spo lys trpC*	143, 227
spoIIIA[c]		P4, Y9, X3, W12		≤54 *lys-1*; 35 *trpC2*		49
		7Z		33 *lys-1*; 16 *trpC2*		148
spoIIIB		A3		56 *lys-1*	*spo lys trpC*	227
spoIIIC[d]		94U		25 *phe-1*; 10 *ilvC1*		148
spoIIID		83U, 25U	RI = 0.1	12 *hisA1*		148, 250
spoIIIE		NG1.67, NG8.17, 82	RI ≤ 0.2	47 *ura-1*; 1 *metC3*	*spoIIG furA cysC spoIIIE*	143, 227, 334
spoIIIE[c]		24T		12 *ura-26*		148
		Y10		16 *ura-1*; 49 *metC3*		49

TABLE 1—*Continued*

Locus	Previous designation (if any)	Allele no. (or strain isolation no.)	Linkage[a]		Order from multiple-factor crosses	References[b]
			Transformation	Transduction (%)		
spoIVA		NG17.23, P20	RI = 0.07 33% *trpC2*	93 *trpC2*	*lys spo trpC*	49, 227, C
spoIVB		P7		66 *lys-1*; 38 *trpC2*	*spo lys trpC*	49
spoIVC		W3, X2, Z7, Z28, E13, E31	RI ≤ 0.24	≤25 *phe-12*; 4 *lys-1*		49, 143, 227
spoIVD		92	RI = 0.89, 0.93 with *spoIVC*	30 *phe-12*; 12 *leu-8*	*leu phe spo*	143
spoIVE		11T		47 *phe-1*; 3 *lys-1*		148
spoIVF		X8, Z5, Z33A, 88	RI < 0.1, 65–80% *phe-12*	97 *phe-12*; 70 *leu*	*leu spo phe*	49, 143
spoIVG		A8, E33	No recombination	47 *argC4*		227
spoVA		89		61 *lys-1*		143
spoVB		91	18% *phe-12*	84 *phe-12*; 51 *leu-8*	*leu phe spo*	143
spoVC		Z10A		70 *cysA14*		49
spoVC[c]		285		79 *cysA14*		B
spoVD		W10		46 *ura-1*; 19 *metC3*		49
spoVE		W5, 85	RI = 0.08 with each other, 0.38–0.61 with *spoVD*	55, 83 *ura-1*; 11 *metC3*	*spoVE spoIIG fur cysC*	49, 143, 334
spoVF		DG47		Unlinked to markers to which *spoVA-E* are linked		49
spo-5NG		5NG		88 *metB10*; 27 *lys-1*		244
spo-W11		W11		24 *phe-12*; 1 *lys-1*		49

[a] Linkage with vegetative markers is expressed as % cotransfer. Linkage between *spo* mutations is expressed as the recombination index (RI [170]).

[b] A, J.A. Hoch, personal communication; B, M. Young, personal communication; C, P.J. Piggot, unpublished observations.

[c] Assignment to locus is not certain, see text.

[d] If 94U (reference 148) is the same as 94 (reference 257), then this has the same phenotype as *spoIVC* mutants described by Coote (49, 50), and the mutations should be placed in the *spoIVC* locus.

III, seven stage IV, and five stage V loci. The different loci are described in detail in the Appendix. The genetic data for this characterization are summarized in Table 1, and the map positions of the *spo* loci are shown in Fig. 2. The primary biochemical lesion is not known for any of the *spo* loci, but is presumed to be in a sporulation-specific function, since the mutants

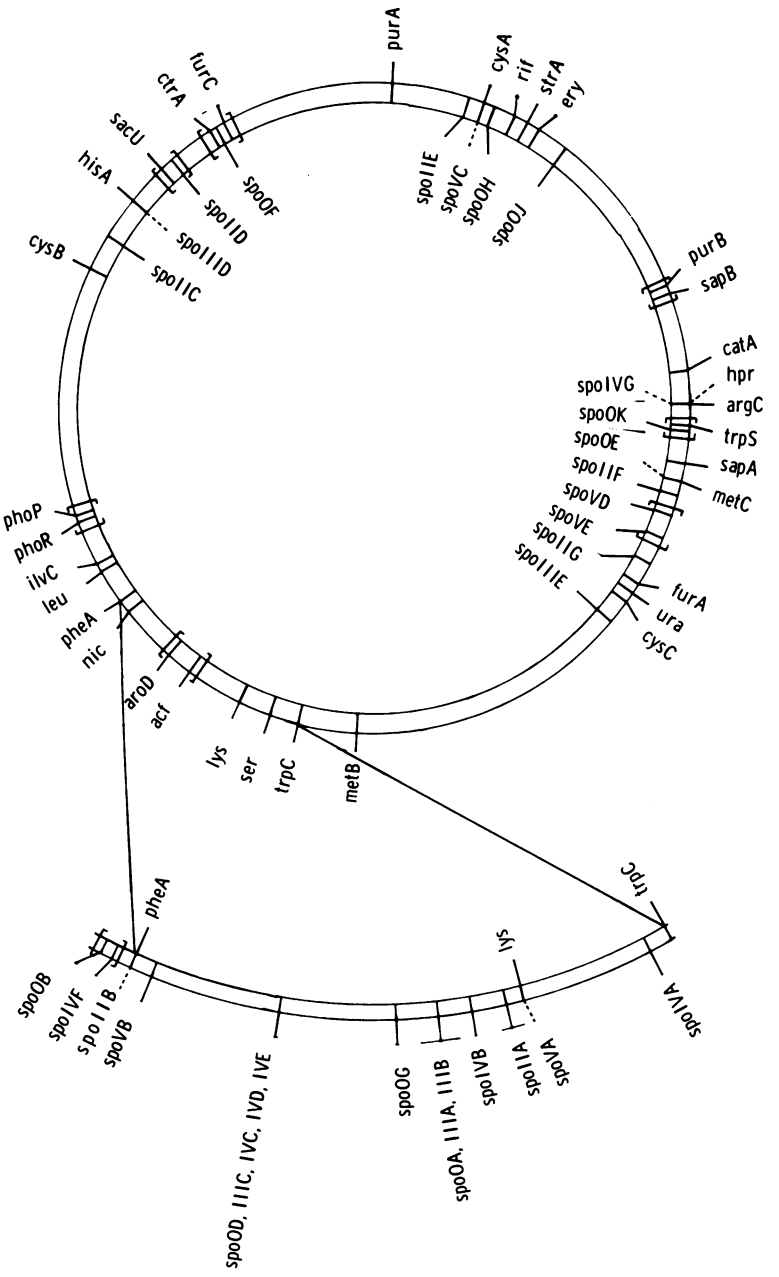


FIG. 2. *Genetic map of the sporulation loci of B. subtilis. The format is based on previously published genetic maps of B. subtilis (182, 332). From published data, the total circumference of the circle is approximately 22 PBS1 transduction units (70, 135, 182, 251, 331). The positions of loci on the map may not be exactly to scale: precise linkage data, together with the appropriate references, are given in Table 1 and in the text. In the expanded map of the pheA to trpC region, the groups of spo loci where orientation relative to each other has not been determined are shown as mapping at a single site. Where no orientation is known, the established linkage is indicated by a dotted line. Map positions placed in parentheses have not been ordered relative to outside markers.*

are not affected in vegetative growth. However, this is an operational definition (see section on nomenclature) and it may be that some of the lesions are in fact in vegetative functions. This reservation applies more strongly to stage 0 than to other loci. The many known lesions in vegetative functions that can block spore formation almost always do so at stage 0 (87). The two types of lesion that can lead to a later block—stage II in a mutant lacking glycerophosphate dehydrogenase (219), and stage V in two mutants requiring glucosamine (93)—do so only in rather special media, and this distinguishes them from all the *spo* mutations that cause blocks after stage 0.

It should be noted that the list of *spo* loci is not complete. Phenotypes have been described that are not represented in the above description (23, 213, 329); since the mutations have not been mapped, they have not been included in the list of loci, but some, at least, could represent mutations in loci other than those listed above. Hranueli et al. used a statistical approach to attempt to estimate the total number of *spo* loci (143). They isolated 16 *spo* mutants at random and mapped the mutations to see whether they were located in any one of 23 known sporulation loci (for our present purposes "locus," as defined here, is approximately equivalent to operon as defined by the authors). Ten of the sixteen mapped in previously described loci, and the remaining six in new loci. From this, total number of loci was calculated [23 (16/10)] to be 37, with 68% confidence limits of 31 and 46. However, this calculation assumed that all loci were equally mutable and that the mutants were equally detectable. When allowance was made for some deviation from these assumptions, a revised estimate of 42, with 68% confidence limits of 33 and 59, was obtained. The authors estimated that to identify all *spo* loci for *B. subtilis* would require the characterization of nearly 2,000 mutants, a daunting task.

There are, in addition, other types of mutant whose phenotypes do not allow the mutation to be conveniently classified in the above loci. Nevertheless, they may be specific for sporulation functions. An oligosporogenous mutant (W11) with a very distinctive phenotype has been described (49), in which the cells seemed to contain compartmented protoplasts surrounded by coat layers; the mutation was linked to *phe-12* and *leu-8* (48% and 22% cotransduction respectively). Balassa et al. (21, 22) have described two unusual types of mutant. The first type had attenuation properties; i.e., each step in sporulation was passed only with low probability. The second type was called late abnormal derepressed (Lad). In the latter, the spore inner coat had five to six layers instead of the usual three, and the mother cell cytoplasm contained inclusion bodies, probably consisting of spore coat fragments. The lesions responsible for these two phenotypes have not been mapped. Hoch and Spizizen (137) and Higerd et al. (129) have described mutants that are hyperproducers of both the serine- and metalloprotease; the *hpr* mutations were up to 32% cotransducible with *argC4* and 10% with *metC1*, but their orientation relative to these markers was not determined. The *hpr* mutations did not impair spore formation (137). Other types of mutant are discussed in later sections.

Frequency of Mutation in Different Loci of *B. subtilis* 168

There is wide variation in the numbers of mutations in the different *spo* loci of *B. subtilis*, ranging from more than 60 in *spo0A* to a single mutation in about half the loci. This is a reflection of several factors. (i) In some studies, mutants were classified only by their ability to form protease and antibiotic; a large number of Pr^+ Ab^+ mutations were mapped (137, 244) but, as the stage at which they were blocked was not determined, they have not been assigned to specific loci here. (ii) Much work has been concentrated on mutations in certain stage 0 loci, and indeed these have been selected specifically in some studies (153, 203, 204, 205). (iii) There may be mutational "hot spots." This is suggested by the work of Rogolsky, who mapped all of a collection of 21 acriflavine-induced *spo* mutations in a single locus (244).

Thus, the relative abundance of *spo0* mutations does not necessarily indicate that stage 0 is more complex than the other stages. When a group of 53 mutants was examined that had been picked at random on the basis of pigment formation, no bias towards stage 0 was apparent (143); in fact, it was the *spoIIE* and *spoIIIA* loci that had a disproportionate number of mutations.

Fine-Structure Mapping of *spo* Loci

Analysis of genetic fine structure in *B. subtilis* requires, in general, crosses by transformation. Where there is no linked vegetative marker, three-factor crosses between *spo* mutants are technically difficult and have not been reported. Recombination indexes obtained from two-factor crosses might be expected to indicate an order of mutations within a locus. Many crosses of this type have been described (see Table 1). However, there is usually too much variation for a map order to be deduced, and

this has only been attempted for the *spoIIIA* locus (227). Where *spo* mutations are cotransformed with vegetative markers, the situation is much more amenable to analysis, Such linkage has been known for some time between *spo0B* and *pheA*, and between *spoIIA* and *lys* (149). Hoch and Mathews took advantage of this to establish a fine-structure map of the *spo0B* locus by three-factor crosses (136). A number of other *spo* loci have now been linked to vegetative markers: *spoIVA* to *trpC* (227), *spoIVF* and *spoVB* to *pheA* (143), *spo0F* to *ctrA* (J. A. Hoch, personal communication), *spo0H* and *spoIIE* to *cysA* (227), and *spo0K* to *trpS* (J. A. Hoch, personal communication). It should prove possible to perform similar analyses for these. The rapid advances in the knowledge of restriction enzymes provide a further technique: the effect on the linkage between markers of the action of *Eco*RI on transforming DNA has already been used to map some restriction sites (125). It may prove possible to extend this technique to map *spo* mutations relative to restriction sites, and hence establish a fine-structure map of the *spo* mutations.

In the initial transformation crosses no case was found of linkage between mutations that caused blocks at different stages of sporulation (250). It is now clear that such linkages do occasionally occur: Young has shown linkage between *spoVE* and *spoIIG* (334); both *spo0B* and *spoIVF* are cotransformable with *pheA*, and on the same side of *pheA* (136, 143), and so they would be expected to be linked to each other by transformation.

Sporulation Loci in Other Species of Endospore Formers

Systems of genetic enchange have been described for several other species of *Bacillus* (for review, see [331]) and for *Thermoactinomyces vulgaris* (142). Sporulation mutants have been described for several of these. However, mapping of *spo* mutatations has only been reported for *Bacillus licheniformis* 9945A (245) and for *Bacillus pumilus* (P. Lovett, personal communication, quoted in [331]). These have not approached the volume of the studies of *B. subtilis*, but both indicate a cluster of *spo* mutations linked to a *lys* marker. For *B. licheniformis*, there were at least four additional clusters (245); the cluster linked to *lys* included a Pr^- mutation.

INITIATION OF SPORE FORMATION

With a good supply of carbon and nitrogen sources, bacilli grow vegetatively and sporulation is repressed; deficiency of either of these may allow spore formation (111, 272). Schaeffer and his colleagues suggested the working hypothesis that sporulation was repressed by nitrogen-containing metabolite(s) whose intracellular concentration(s) depended on the rate of metabolism of the available carbon and nitrogen sources (272). Sporulation may also be initiated by phosphate starvation (111), suggesting that the repressing metabolites might be phosphorylated. The hypothesis did not imply an all-or-none response; even in rich media there was a low probability that a cell would sporulate, and this probability was greater in poorer media (272). Studies of spore formation in chemostat cultures established that there was a continuous range of probabilities and that the incidence of spore formation in a particular medium was a function of the growth rate (60, 61). The results confirmed that sporulation could occur at high frequency with limitation of carbon or nitrogen source, but not with limitation of auxotrophic requirements or essential cations.

It has been generally assumed that the repression of sporulation is in many ways, similar to the catabolite repression of inducible enzymes that is also brought about by the presence of easily degradable carbon or nitrogen sources. Various studies have looked for evidence of this, and it now seems clear that different mechanisms operate to overcome the two types of repression (see below). Nevertheless, in several cases, analagous compounds have been shown to be involved in the two phenomena.

It may be easy to visualize molecular models for initiation, but before considering these, it is as well to emphasize that it is difficult to identify precisely the time of initiation in different experimental systems. When induced by exhaustion, sporulation is generally assumed to begin at the end of exponential growth. However, Kretschmer found that the time between the end of growth and the formation of the spore septum varied considerably between different media (167), suggesting that it might be difficult to define a precise initiation point. Moreover, biochemical changes that may be necessary for sporulation take place before the end of growth (63, 168). Stage 0 mutants begin to differ from the wild type before the end of growth (35, 36, 206), although in this case it could be argued that the mutations are not specific for spore formation. The point remains that changes take place before the end of growth, and these affect sporulation. The exact time of the end of exponential growth is difficult to estimate in exhaustion systems, and these apparent anomalies may reflect this imprecision. There is no evidence for changes dur-

ing growth before sporulation when sporulation is induced by replacement of a rich with a poor medium. However, variation in the amount of rich medium "carried over" affects the timing of later sporulation events (P. J. Piggot, unpublished observations) and so, presumably, affects the timing of initiation and confuses its identification.

In addition, it is clear that events before the final symmetrical (vegetative?) division influence the course of spore formation. In pairs of sister cells, the location of the sporulation septum in each cell is not random with respect to the other cell (reviewed in [87]). This position is influenced by the media used (131; J. Mandelstam, personal communication), and so is unlikely to be determined solely by the position of old and new growth zones of the vegetative cell wall. In populations of wild-type *B. subtilis* exhibiting several of the stages of sporulation, there is a highly significant tendency for sister cells to be at the same (59). This indicates that either sporulation was initiated before the final symmetrical division, or some event occurred before the final symmetrical division ensuring that, if initiated, the daughter cells would be initiated together (59). The event could be DNA replication, but other explanations are possible.

Possible Effectors

Metabolites that repress sporulation. Freese and co-workers have attempted to define the biochemical nature of sporulation repressors by using mutant strains in which the rapid metabolism of available carbon or nitrogen sources was restricted. They have found that compounds entering a number of metabolic routes, where subsequent metabolism is blocked by mutation, can suppress sporulation (87, 89, 91). Thus, a mutant lacking glycerol phosphate dehydrogenase accumulated α-glycerol phosphate to such an extent that sporulation was inhibited, although a low level of this compound was necessary for spore formation, presumably as a membrane precursor (89, 219). A triple mutant that lacked the enzymes needed to metabolize glucose 6-phosphate accumulated this compound in the presence of glucose, and spore development was arrested before septation (91). A strain lacking phosphoglycerate kinase was unable to sporulate in the presence of excess malate, which entered the metabolic routes below the mutation. Thus, malate, or some compound derived from it, such as pyruvate or L-alanine, could suppress sporulation. In addition, rapidly metabolizable carbon sources of higher molecular weight, such as glucose, that entered metabolic routes above the block also suppressed sporulation. This indicated that compounds in either subdivision of the metabolic routes could suppress sporulation (91). These studies have thus identified a number of metabolites that, when accumulated by cells, are able to suppress sporulation before stage II. These compounds would normally be present in fairly low concentrations during growth, and it remains an open question as to whether their depletion would ordinarily play a role in the initiation of sporulation.

This approach, involving the analysis of mutants with known enzymic defects, does establish that a metabolite such as α-glycerophosphate has to be provided at a precise intracellular concentration for sporulation to occur. This may be different from the concentration required (or permitted) for vegetative growth (89). However, the work has not, as yet, pinpointed the initial biochemical reactions required to set the sporulation process in motion. If anything, it suggests that there may be no single initial metabolic reaction.

It seems clear that sporulation requires an active system for producing ATP, and that the tricarboxylic acid cycle fills this role (88). It has also been reported that the end of exponential growth in *B. subtilis* is accompanied by a fall in the ATP level in the cell, which was shown to be transient in one report (88), but more prolonged in another (144). The latter authors showed that in all instances where ATP levels were maintained at the level found in growing cells, sporulation was prevented. They suggested that the change in ATP level, or in total adenylate energy charge, brought about by starvation of an available energy source might be the signal that initiates sporulation; phosphorylation or adenylation of an aporepressor protein would be a possible mechanism for effecting the repression. After this initial signal the ATP level, or adenylate charge, would then be restored. The hypothesis should be easy to test: replacement of air by nitrogen in a growing culture led to a 10-fold fall in ATP concentration in less than 1 min (88); it might be anticipated that brief exposure of growing cells to an oxygen-free atmosphere would result in the initiation of sporulation. A similar experiment, subjecting *B. subtilis* growing in broth to a brief anaerobic shock, has been performed, but no derepression of sporulation was observed (267). This experiment might be better performed with bacteria growing in a minimal medium, since cells induced to start sporulating in broth would tend to resume growth rather than go on to form mature spores (see section on Commitment).

Compounds that appear at the initiation of sporulation. The intracellular concentration of 3'5'-cyclic guanosine 5'-monophosphate (cGMP) rises sharply at the start of spore formation (31), suggesting that it might have some role in the initiation of the process. In gram-negative bacteria 3'5'-cyclic adenosine 5'-monophosphate (cAMP) has been shown to be intimately involved in the catabolite repression of inducible enzyme synthesis (28, 41, 78, 225, 233), and addition of exogenous cAMP can affect the rate of β-galactosidase synthesis by *Bacillus megaterium* (316). However, neither cAMP nor the associated cyclase and phosphodiesterase activities have been detected in any appreciable amounts in *Bacillus* species (31, 146, 273), so that it is unlikely to have any role in vivo. It seemed reasonable to speculate that the structurally analagous cGMP might play some role in the catabolite repression of inducible enzyme synthesis in bacilli and in the catabolite repression of sporulation. However, attempts to isolate *Bacillus subtilis* mutants defective in catabolite repression were not successful (51), and no mutants defective in cGMP metabolism have been reported, so that it has not been possible to test the speculation.

Rhaese and co-workers (240) have characterized a series of highly phosphorylated nucleotides (HPNI to IV) that appear at the start of sporulation. The authors have deduced the structures ppApp for I, ppAppp for II and, more tentatively, pppAppp for IV and ppZpUp for III (where Z is an unknown sugar). These are structurally analagous to the nucleotides MS1 (ppGpp) and MS2 (pppGpp) that accumulate on amino acid starvation of *Escherichia coli* (44) and also *B. subtilis* (97). In this context, it is particularly interesting that the "idling" reaction of ribosomes from exponentially growing *B. subtilis* produced MS1 and MS2, but the same reaction using ribosomes and ribosomal wash from sporulating cells produced HPNI and HPNII (241). The situation is clearly complex, as ribosomes were not required for the synthesis of HPNIV (241a). There are now several reports demonstrating that the MS nucleotides are not involved in sporulation (84, 240, 268). In contrast, comparable experiments continued to associate the appearance of HPNs with the onset of sporulation. Thus, HP, (241a) but not MS (268), nucleotides accumulated when spore formation was triggered by phosphate starvation, whereas a relaxed mutant that could not produce MS1 and MS2 did produce the HP nucleotides and sporulated normally (240).

There is, then, a strong case for the involvement of the HP nucleotides in sporulation. However, the precise nature of this involvement remains to be established. It is not clear, for example, whether they are produced immediately upon initiation or whether they accumulate as a result of the onset of sporulation, perhaps reflecting a functional change in the translational machinery, as has been suggested by Hanson (119). It has been shown that MS1 stimulates enzyme synthesis (54), and it is possible that, by analogy, the HP nucleotides may promote the expression of sporulation-specific genes. There are no reports of any mutants that are the HPN equivalent of relaxed mutants, nor of the behavior of early-blocked sporulation mutants with respect to HPN synthesis.

Glutamine synthetase. In enterobacteria, glutamine synthetase (GlnS) has a critical role in the regulation of the synthesis of certain enzymes by the availability of nitrogen (194, 233, 234, 315). Aubert and colleagues have investigated the possibility that GlnS might have a similar role in the initiation of sporulation. They isolated mutants of *B. megaterium* that were defective in GlnS (74, 75, 239). These were often deficient in their ability to sporulate, but no consistent pattern was observed. There was no correlation between the level of sporulation and the GlnS activity or the amount of GlnS protein assayed immunologically. Thus, the details of the suggested regulatory role remain to be established.

Elmerich and Aubert also found that a GlnS-negative mutant (*gln⁻-26*) was able to sporulate efficiently in a medium containing glucose, NH_4^+, and glutamate, whereas the wild type, and a mutant lacking glutamate synthase, sporulated very poorly in this medium (75). A revertant of *gln⁻-26* regained all the wild-type properties. Further studies suggested that it was not glutamine itself, but rather a component in the early part of the purine biosynthetic path that was involved in the repression of sporulation (76, 77). The authors speculated that glutamine synthetase would be the receptor for this effector.

Relationship to DNA Replication and the Cell Division Cycle

There is now strong evidence that initiation of sporulation is tied to the cell cycle (60, 61, 254), and can only occur while DNA is being replicated (59, 73, 198, 200, 217). When chromosome replication of a thymidine-requiring mutant was prevented by thymidine starvation, then this prevented the production, in a sporulation medium, of serine protease and all the subsequent sporulation events tested (56). Thus, the susceptible period of DNA replication is before (and necessary for) an event that is

regarded as one of the earliest sporulation events. It seems reasonable to consider the requirement for DNA replication as a requirement for the initiation of spore formation, rather than a later event in the process.

In exponentially growing cultures of *B. subtilis*, termination of DNA replication starts a round a membrane protein synthesis that eventually leads to the formation of a division septum (264, 265). It is plausible that chromosome replication must terminate in a sporulation medium for a sporulation, rather than a division, septum to be programmed. Mandelstam et al. found that, when a thymidine-requiring mutant was induced to sporulate by a replacement technique, thymidine had to be present for at least the first 90 min in the sporulation medium to obtain maximum sporulation (200). This was consistent with a requirement for termination of replication. Experiments, in which 6-(*p*-hydroxyphenylazo)uracil was used to block DNA replication of *B. subtilis* wild type, have also indicated that there is such a requirement (G. Dunn, personal communication).

Mandelstam and co-workers have provided evidence that there is also a requirement for the replication of an early part of the chromsome in a sporulation medium in order to induce spore formation. They made use of a mutant of *B. subtilis* (*ts-134*) that was temperature sensitive for the initiation of DNA replication to obtain cultures in which the population was undergoing a single round of synchronous DNA repliction. The capacity for sporulation was initially low in the samples, reached a peak about 15 min after DNA replication had started, and then declined (73, 198). The experimental technique for inducing sporulation of this mutant required some carry-over of rich medium into the poor sporulation medium (73), and so it is difficult to give a precise time to the initiation of sporulation. However, the period of peak susceptibility to spore formation corresponded roughly to the time at which the *spo0H* and *spo0J* loci would be replicated (198). Thus, it is possible that expression of these loci could only be activated while they are being replicated (198). This could be mediated by one or more of the effectors discussed earlier. Whatever the explanation, the requirement for replication of an early part of the chromosome appears to be distinct from, and comes earlier than, any requirement for chromosome termination in the sporulation medium.

The requirement for DNA replication in order to start spore formation is an unusual control mechanism. It contrasts with the observation that inhibition of DNA synthesis by thymidine deprivation did not prevent the induction of such enzymes as sucrase (51). This indicates that even though the repression of sporulation and of enzyme induction may involve some common catabolite repressor, a fundamental difference exists between the two processes with regard to chromosome replication. The obvious analogy to spore formation is with cell division. Indeed, the initial stages of sporulation are usually regarded as a modified cell division: the starvation conditions promote unbalanced metabolism, one result of which is the asymmetrically sited sporulation septum. It is not within the scope of this review to discuss the analogy with cell division in further detail (see [87, 132, 167]). However, certain points bear on the discussion of the genetics of sporulation.

It has been suggested that, at the start of spore formation, there is a signal to prevent the formation of an incipient vegetative division septum and a signal, which may be separate, to form a sporulation septum at the pole of the cell (72). Either or both signals could be controlled by chromosome replication. There are several reports that some stage 0 mutations lead to "normal" length bacteria in sporulating conditions, whereas other stage 0 mutations lead to bacteria of half the "normal" length (23, 49, 72, 168). Dunn et al. postulated that the "short" stage 0 mutants had an earlier block and that they did not receive the signal to stop a last symmetrical division. In confirmation of this, a double mutant harboring the two types of stage 0 mutation formed bacteria that were half normal length (72). Kretschmer and Fiedler had earlier noted that stage 0 mutants of *B. megaterium* that were capable of forming short cells did so only in certain media (168). For the model of Dunn et al. to fit this observation, the loss of the signal not to form a division septum must presumably depend on the medium. It will be interesting to see how such models extend to abortively disporic mutants. Have these mutants somehow suppressed too many symmetrical divisions? Are the two polar septa formed simultaneously or sequentially? During the vegetative cell cycle of *B. subtilis*, the rate of cell length extension is porportional to the number of growth zones, and these can be regarded as the incipient sites for septum formation (265). Knowledge of the changes in the rate of length extension during sporulation may, therefore, provide evidence for the pattern of incipient septum formation during spore formation.

The initial changes associated with chromosome replication are presumably mediated by proteins that bind to DNA. There are now several reports of changes in the pattern of DNA-

binding proteins during sporulation (35, 242; H. A. Foster, personal communication). The pattern of soluble DNA-binding proteins from stage 0 mutants was reported to be different from that of the wild type, even in extracts prepared from growing cells (36). The authors suggested that this might represent a subtle difference in the normal cell cycle that subsequently prevented spore septum formation. However, as yet, no specific function in spore formation has been demonstrated for any of the protein changes. Indeed, one might anticipate that any protein that did link DNA replication to septation would be membrane bound, and so might not have been present in the soluble fraction studied. There are no reports of the pattern of proteins from *spo* mutants blocked after septation.

Mutations Affecting the Initiation of Sporulation

Hyper-repressed. Bacilli can grow vegetatively in media with poor nitrogen or carbon sources. However, a significant portion of the population will be sporulating rather than growing (60, 272). In these circumstances, a mutant that cannot initiate sporulation will be growing faster than the wild type because the whole population of the mutant will be growing vegetatively. A similar argument applies to other situations, such as continuous culture, in which there is a possibility of growth as well as sporulation (10, 11, 12, 60, 61). The argument does not depend on any particular mechanism for initiation. Mutants that are blocked after the initiation of sporulation might also have some advantage over the wild type, depending on how readily the bacteria that had started to sporulate could resume vegetative growth; they would be expected to have a disadvantage with respect to initiation mutants.

Michel et al. successfully adopted this approach to isolate a large number of stage 0, and only stage 0, mutants without prior mutagenesis (204, 205). They used shifts-down in nitrogen (NH_4^+ to nitrate) or carbon (glucose to citrate or histidine) source followed by prolonged growth. The effect was most striking with the nitrogen source (204): 16 cultures were grown up from single wild-type colonies. After about 20 generations in the nitrate medium, 50 to 100% of the bacteria in every flask were stage 0 mutants. Several of these were characterized further. Most had the earliest stage 0 phenotype (Pr^- Ab^-), whereas the rest had the $Pr^\pm$ Ab^- phenotype. Ten of the 11 Pr^- Ab^- mutants examined had single mutations mapping in the *spo0A* locus (203). The poor carbon sources were not as effective as the poor nitrogen source. After 30 generations, *spo* mutants usually represented, at the most, only a few percent of the population (citrate was more effective than histidine). Although the mutants were all blocked at stage 0, there was no preponderance of *spo0A* mutants (205); this pattern resembled that of cultures grown for only a few generations in nitrate after a shift-down from NH_4^+.

Stage 0 mutants were also found to accumulate when cultures that had sporulated in a rich medium were kept aerated for a long time after sporulation was completed (205). The increase in the mutant population was most striking after autolysis of the sporangia; this presumably supplied nutrients for growth of the mutant organisms. The same selective mechanism could again be invoked.

Thus the work of Michel and collaborators has indicated that the *spo0A* locus in particular may be involved in the very earliest events of the initiation to sporulation, and that *spo0A* mutants are unable to initiate sporulation. This would be consistent with the phenotype exhibited by many of the mutations placed in this locus, which give rise to the most pleiotropically negative of all the sporulation phenotypes (37, 203). The locus has been studied in detail, and well over 60 distinct mutations are known. Recomination indexes from transformation crosses indicate that they are located in, at the most, two or three adjacent genes (133, 143, 203; J. A. Hoch, personal communication). The Pr^- Ab^- *spo0A* mutants may reasonably be regarded as hyper-repressed for sporulation. However, the suggestion that *spo0A* mutants might also be hyper-repressed for catabolite repression of inducible-enzyme synthesis was found not be the case (37, 267), since the mutants were able to produce such catabolite-repressible enzymes as histidase and α-amylase. In fact, histidase was less sensitive to catabolite repression by glucose in a *spo0A* mutant (37).

The studies of Hoch (133) indicated that either a missense or a nonsense mutation in the *spo0A* locus could give rise to the pleiotropic Pr^- Ab^- phenotype. This indicated that the locus, at least the part where the mutations were located, was translated into protein. Hoch suggested that this indicated a positive control mechanism in which the product of the *spo0A* locus was required to induce subsequent sporulation events. Karmazyn et al. (157) and Broadbent (personal communication) have shown that *spo0A*/*spo*$^+$ merodiploids for four Pr^- Ab^- *spo0A* mutations (out of four tested) showed segregation patterns that were consistent with the *spo0A* mutations being dominant over the wild type. This is not immediately compatible with positive control. It has been suggested

that the product of the locus might be a membrane component (113, 151), and this would fit more readily the merodiploid data. However, it does not fit easily with the case in which there was evidence that a single mutation was both suppressible by nonsense suppressors and dominant to the wild type (157). In view of the complex nature of the locus and the many pitfalls in interpreting diploid analyses (29), it would seem premature to draw any far-reaching conclusions. Despite strenuous efforts, the primary product of the locus has not been determined (37, 112). Perhaps the most pressing need is a comprehensive fine-structure genetic map that orders the different types of mutation within the locus. It is of interest that the segregation pattern of the one Pr^+ Ab^+ *spo0A* mutation tested in merodiploids indicated that it was recessive to the wild type (158); no studies have been reported for the suppression pattern of this type of mutation.

Derepressed. The *spo0A* mutants may be regarded as a type of initiation mutant that is uninducible, or hyper-repressed, for sporulation events. An alternative type of initiation mutant would be one that had a derepressed sporulation phenotype insensitive to the presence of utilizable carbon, and/or nitrogen, sources. A number of such derepressed mutants have now been described (3, 77, 154, 169, 183, 296). Some are purine auxotrophs, and many are hyperproducers of protease. However, there are several distinct types so that the situation is not comparable to that with the hyper-repressed mutants. In the cases where the mutations in the derepressed mutants have been mapped, they are quite separate from any *spo* loci. Thus, there is no reason to suppose that mutations in any one locus can give rise to both uninducible and derepressed mutants.

An early report of a derepressed mutant concerned a strain of *B. subtilis* that was able to sporulate, at a level of about 10% of the bacterial population, during exponential growth in media with glucose or glycerol as carbon source, but not with sucrose as carbon source, nor in broth media (296). The strain was isolated after repeated exposure of cells to ultraviolet irradiation and was apparently not investigated further. Several mutants of *B. cereus* T have been described by Levisohn and Aronson that were able to sporulate massively in an amino acid mixture which inhibited sporulation of the wild type (183). The mutants tested could also sporulate in the presence of a concentration of glucose that was inhibitory for sporulation of the wild type. The mutants produced high levels of extracellular protease; several were also purine auxotrophs. In one case, prototrophic revertants of a purine auxotroph were isolated and shown to have also reverted to the wild type with regard to sporulation and extracellular protease production, so that the pleiotropic effects were caused by a single mutation. In a later study, Aronson and co-workers (3) isolated a number of hypoprotease producers, many of which were also purine, or pyrimidine, auxotrophs; however, these strains apparently formed spores in the normal way. Elmerich and Aubert (77) have recently described purine auxotrophs of *B. megaterium* that were also able to sporulate under conditions where the wild type could not. They suggested that a component of the early part of the purine pathway might be involved in the repression of sporulation (see section on Possible effectors).

Ito and Spizizen have reported mutants of *B. subtilis* that were able to sporulate in the preence of glucose or an amino acid mixture (154). The mutations fell into two groups. One group, designated *catA*, caused the production of five to six times more exoprotease than did the wild-type allele. The other group, designated *catB*, did not affect exoprotease production. One of the *catA* mutants was studied in detail. The *catA* mutation was located between the *tre* and *metD* markers on the genetic map (154) and was quite distinct from any known *spo* locus; insensitivity to glucose repression of sporulation was inseparable from hyperprotease production in genetic crosses involving the *catA* mutant (154). The mutant grew normally on glucose and, in addition, the induction of histidase was repressed by glucose in the normal manner. Therefore, the ability to overcome glucose repression of sporulation was not caused by an impairment of metabolism of glucose, nor by a defect in the general catabolite repression mechanism (154). Thus, as with the presumed hyper-repressed *spo0A* mutants, the lesion was apparently specific to sporulation-associated functions.

Kunst et al. have described a type of mutation ($sacU^h$) in *B. subtilis* that resulted in hyperproduction of extracellular levan-sucrase, hyperproduction of exoprotease, and an ability to sporulate in the presence of glucose or an amino acid mixture (169). Some of the $sacU^h$ mutations also caused a loss of motility. The authors extablished that this complex phenotype resulted from a single mutation. The *sacU* locus mapped quite separately from *catA* (182).

These studies have revealed that several types of mutant exist (isolated by widely different procedures) which combine the hyperproduction of exoprotease with the ability to sporulate under conditions where the wild type is unable to do so. Several other hyperprotease-

producing strains of *B. subtilis* have been described (16, 22, 129, 137), and it would be interesting to know whether these are also altered in their ability to sporulate.

Sporulation of the wild type can be stopped for some time after its initiation by the addition of rich media (commitment to spore formation is discussed more fully later, as it is essentially a phenomenon of subsequent sporulation events rather than initiation). Thus, it is possible that in very rich media mutants able to initiate sporulation would not be able to complete the process and so would not be detected. That the mutants described in this section were able to sporulate at all in relatively rich media (which repressed sporulation of the wild type) indicates that, once initiated, they were committed to sporulate in these media. There are two interpretations of this behavior: (i) the conditionally constitutive initiation mutants have a lesion in some general metabolic reaction that prevents repression of initiation and also the repression of the subsequent stages of sporulation; (ii) repression of initiation is different from the repression of later stages, and in the conditions used to isolate and study the mutants, the later stages were not repressed. It has already been mentioned that the wild type exhibits a continuous range of sporulation frequencies in different media (60, 272); this is more compatible with the second interpretation, but does not rule the first out absolutely.

REGULATION OF SUBSEQUENT SPORULATION EVENTS

Studies of initiation have generally attempted to identify a single critical event that could be said to start the process of spore formation. Such an approach is not suitable for the study of the rest of spore formation where there is no a priori reason to suppose that any one event is more critical than the rest. Consequently, the emphasis is more on sequences of events, their organization and control, with correspondingly less emphasis on any one molecular mechanism. In the following sections we consider various approaches that have been made toward an understanding of how the expression of sporulation loci might be organized. As befits a genetical analysis, heavy emphasis is placed on studies of mutants. It is as well to emphasize at the outset that in almost every case the immediate role of a mutated gene is unknown.

Dependent Sequence of Events

Several new biochemical activities appear at characteristic times throughout the sporulation process (see section on Morphology and biochemistry of sporulation). They can be correlated with a particular morphological stage of development. In general, asporogenous mutants that are blocked at a particular morphological stage undergo all the biochemical changes associated with the previous stages, but show none of the biochemical changes associated with stages after the block (49, 320). This pattern is evidence for the relevance to sporulation of certain biochemical changes for which a functional requirement may not be apparent. In addition, the pleiotropic nature of the *spo* mutations (19, 266, 271, 286, 320) suggests the presence of a primary dependent sequence of events, whereby a later event will only occur after the successful completion of earlier events (19, 320).

The sequence of changes involves the expression of all the known *spo* loci (Table 1). Mutations defining these loci are not, in general, expressed during vegetative growth, but only during sporulation. Thus, there are presumably signals for "switching on" expression of the loci. As most of the loci are widely separated on the genome, it must be presumed that a diffusible substance is generally responsible for this. The most acceptable model for a temporal process such as sporulation involves the sequential activation of the relevant loci (see [118, 289]), whereby one or more of the products resulting from the activity of a particular locus would be responsible for turning on expression of the next locus. This could be a protein product of the locus, or it could be some other molecule resulting from the action of an enzyme that is a product of the locus. A further consequence is that each locus that does map separately from the other loci must have its own control elements in addition to any structural genes. For this reason such loci, of which more than 30 are now known, have been described as sporulation operons (227).

Order of Expression of Loci from Mutant Phenotype

As there is a close correlation between biochemical events and morphological changes during sporulation, it would seem reasonable to deduce the order of expression of *spo* loci merely by reference to the phenotype of *spo* mutants. A series of biochemical events is associated with the earliest stages of sporulation, and a number of these have been used to describe *spo0* mutants (Table 2; see section on Sporulation loci). Although there is some variation between laboratories in scoring protease and antibiotic production, a hierarchy of pleiotropy can be formulated, starting with the *spo0A* mutations that resulted in the most completely pleiotropic phenotype:

TABLE 2. *Summary of the properties of spo0 mutants*[a]

Locus	Character[b]				
	Cpt[c]	Pr	Pha	Cyt	Ab
spo0A	−[d]	−	−	−	−
	+[d]	+	ND	ND	+
spo0B	+	±	−	−	−
spo0G	+	−	ND	ND	−
spo0D	+	+	ND	ND	−
spo0E	ND	±	+	+	Var
spo0F	+	±	Var	+	−
spo0H	ND[e]	+	+	+	+
	ND[e]	±	ND	ND	Var
spo0J	ND	+	ND	ND	+
spo0K	ND	+	ND	ND	ND

[a] Appropriate references are given in the text.

[b] Characters are scored as: +, wild type; −, mutant; var, variable; ND, not determined.

[c] Cpt, competence; Pr, extracellular protease formation; Pha, efficiency of plating of $\phi 2$ and $\phi 15$; Cyt, pattern of cytochrome induction during sporulation; Ab, antibiotic formation.

[d] Two types of *spo0A* mutant (see text).

[e] *spo0H* mutants have been scored differently by different laboratories (136, 227; see text).

spo0A → *spo0G* → *spo0B* → *[spo0F, spo0D]* → *spo0E* → *spo0H* → *[spo0J, spo0K]*

A sequence can be deduced for stage II, based on three biochemical events (Table 3), and for stage IV, based on morphology (see section on Sporulation loci):

[spoIIE, spoIIF, spoIIG] → *spoIIA* → *[spoIIB, spoIIC, spoIID]; [spoIVC, spoIVF, spoIVD]* → *spoIVA* → *[spoIVB, spoIVE, spoIVG]*

There is no obvious order for the *spoV* loci, and the *spoIII* loci are not easily distinguishable by phenotype.

This tentative arrangement of loci according to phenotype implies the temporal dependency of expression of a later locus on that of an earlier one within these groups, but it does not establish this for any particular locus. There are certain observations that would prevent any such extrapolations of this approach. Thus, mutations mapping within the *spo0A* locus can give rise to quite distinct biochemical phenotypes (pr^- Ab^-, Pr^+ Ab^+; see description of *spo0A* locus). Had these mutations mapped in different regions of the chromosome, then the loci they represented would have been placed at quite different points in a sequence of loci arranged according to phenotype. The two types of mutation map very close together (it has yet to be established that they lie in separate genes within the *spo0A* locus), and it is at least plausible to assume that they lie in a single operon whose genes would be expressed together. This supposition implies that ordering loci by phenotype may not give the temporal order of their expression.

Deposition of the cortex takes place during stage IV, and the deposition of the coat layers is used as the phenotypic characteristic to define stage V. Yet cortexless mutants have been described which have well-developed coat layers or deposit coatlike material in the sporangium (24, 25, 49, 93, 143, 213, 224). For *B. subtilis*, mutations in the *spoVB*, *spoVD*, and *spoVE* loci affect cortex, but not coat, formation, whereas mutations in the *spoIVB*, *spoIVE* and *spoIVG* loci allow normal cortex formation, but little or no coat formation. This suggests a parallel sequence of events or a branched pathway that would allow apparently later events to occur after a block. Indeed, mutants blocked as early as stage II are able to synthesis material that reacts with antisera to alkali-soluble coat protein (327; Table 3). This is consistent with the observations that cortex and coat synthesis can occur concurrently (82, 221) and that coat deposition may preceed the deposition of cortex in certain clostridia (192, 249). Coat morphogenesis has recently been reviewed by Aronson and Fitz-James (6a).

Epistasis of Sporulation Mutations

In an attempt to define more clearly the order of expression of loci, Coote and Mandelstam (52) investigated the epistatic relationship between a series of pairs of sporulation mutations. Two mutations, each of which gave rise

TABLE 3. *Summary of biochemical properties of stage II and stage III mutants*[a, b]

Locus	Character[c]		
	AP[d]	ASC	GDH
spoIIA	−	+	−
spoIIB	+	ND	−
spoIIC	+	ND	−
spoIID	+	+	−
spoIIE	−	−	−
spoIIF	−	ND	−
spoIIG	−	ND	−
spoIIIA	+	+	+, −[e]
spoIIIB	+	ND	ND
spoIIIE	+	ND	−

[a] Appropriate references are given in the text.

[b] None of the properties listed has been determined for *spoIIIC* or *spoIIID* mutants.

[c] Characters are scored as: +, wild type; −, mutant; ND, not determined.

[d] AP, Alkaline phosphatase; ASC, alkali-soluble coat protein; GDH, glucose dehydrogenase.

[e] Different mutants gave different types.

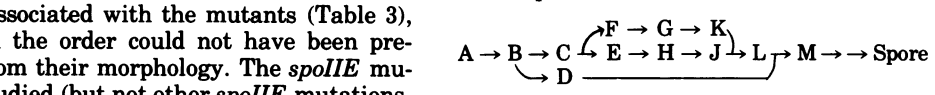
to a distinct sporulation phenotype, were introduced into a single organism and the phenotype of the double mutant was compared with the phenotypes resulting from the two mutations considered singly. If the two genes involved were part of a dependent sequence of gene expression, then the phenotype of the double mutant would be determined by the mutation in the gene that was expressed earlier in the dependent sequence, as this mutation would have prevented the expression of the other mutations. However, if the two genes were not expressed as part of a single dependent sequence, but were expressed independently or had their expression linked in some other way, then the phenotype of the double mutant need not be the same as that of either parent.

In the cases examined where the mutations caused blocks before stage IV, one mutation determined the phenotype of the double mutant (52). This was compatible with a single linear dependent sequence. For stage II, a consistent pattern of epistasis was observed, *spoIIE* → *spoIIA* → *[spoIIC, spoIID]*. This agreed with the presence or absence of the biochemical events associated with the mutants (Table 3), although the order could not have been predicted from their morphology. The *spoIIE* mutation studied (but not other *spoIIE* mutations, see description of the locus) led to the formation of a single septum, as did the *spoIIC* and *spoIID* mutations, whereas the *spoIIA* mutation led to an abortively disporic phenotype. Thus, the epistatic sequence was: *spoIIE* (AP^- ASC^-, single septum) → *spoIIA* (AP^- ASC^+, abortively disporic) → [*spoIIC, spoIID*] (AP^+ ASC^+, single septum).

In a further series of experiments, the epistatic relationship *spoIIIE* → *spoIVA* → *[spoVD, spoVE]* was established. However, similar relationships could not be deduced for all the pairs of late mutations. Mutation in the *spoIVA* locus causes deposition of coat material in the sporganium instead of around the developing spore (49; Fig. 5). When such a mutation was introduced into a strain with a mutation in the *spoIVF* locus, the double mutant showed a modified *spoIVA* phenotype (52); the coat material tended to wrap itself around the prespore in a more normal manner. This phenotype differed from that of either parent—mutation in the *spoIVF* locus alone does not allow any coat deposition. Thus, both mutations influenced the phenotype of the double mutant. This might be expected if the two loci were not part of a single dependent sequence of gene expression. The *spoIVA* locus is presumably concerned in some way with coat deposition, and the sequence of events leading to coat deposition may, once initiated, be separate from the sequence of other sporulation events.

The double mutants with mutations *spoIVF/spoVD* or *spoIVF/spoVE* had a typically stage III phenotype. Again, it is only possible to explain this by assuming that the expression of neither locus is directly dependent on the expression of the other. It is conceivable that several gene products may be required at the same time for the successful transition from, say, stage III to stage IV; failure to produce one of the gene products might allow development part way into stage IV, but failure to produce two products simultaneously would stop all development beyond stage III.

The epistatic relationship of mutations blocked as late as stage III are compatible with (although they do not prove) a single linear dependent sequence of gene activation during sporulation. However, the results, using late-stage mutants, are not compatible with this model; rather, they are compatible with parallel paths of gene activation. This can be visualized in a scheme such as the following, which is a development of an earlier scheme (52):



where A → B indicates that event B is dependent on A. E → H → J and F → G → K are two dependent sequences that act in parallel; successful functioning of both pathways is required to go to L. D is synthesized early, but is not required until much later in the process. Some phenotypes that would result from blocks at various points in this scheme are shown in Table 4. Where there is a single dependent sequence, then the double mutant (e.g., 1 and 2) shows the phenotype characteristic of the earlier mutation (1). Where there is more than one dependent sequence, then the double mutant (4 and 5) appears to be blocked earlier than either single mutant. Event D may be analagous to coat protein formation. Here, early blocked mutants (e.g., 2, 4, or 5), would still synthesize D, and a mutant blocked specifically in the synthesis of D (3) would appear to be blocked at a late stage (L).

Temperature-Sensitive Mutants

The studies discussed in the preceeding sections have given a general idea of the order of expression of *spo* loci. However, the methods do not give the precise time during sporulation that a particular locus becomes active. The process as a whole could conceivably continue for some time after a mutated gene begins to be expressed before finally coming to a halt; in-

TABLE 4. *Phenotypes of mutants and double mutants with lesions in a hypothetical scheme for the sequence of sporulation events*

Mutation	Step blocked	Phenotype												
		A	B	C	D	E	F	G	H	J	K	L	M	Spore
1	A → B	+	−	−	−	−	−	−	−	−	−	−	−	−
2	B → C	+	+	−	+	−	−	−	−	−	−	−	−	−
1 + 2		+	−	−	−	−	−	−	−	−	−	−	−	−
3	B → D	+	+	+	−	+	+	+	+	+	+	+	−	−
4	C → E	+	+	+	+	−	+	+	−	−	+	−	−	−
5	C → F	+	+	+	+	+	−	−	+	+	−	−	−	−
4 + 5		+	+	+	+	−	−	−	−	−	−	−	−	−

deed, such an "unbalanced" development could explain the abnormal phenotypes of many sporulation mutants, and was used to explain the results of certain epistasis experiments. A study of temperature-sensitive (or other conditional), as distinct from unconditional, *spo* mutants, might be expected to go some way toward answering this point. In addition, it might answer additional questions, such as the minimum time that a locus needs to be expressed, whether expression is required at more than one time during sporulation, and the nature of the primary product(s) of the locus. These hopes are beginning to be realized, since several temperature-sensitive (ts) mutants have now been characterized in detail (176, 177, 180, 181, 191, 297, 335). The results are strictly applicable only to those loci studied. They indicate that *spo* loci are expressed in a variety of ways.

The ts mutant with the earliest block in the sporulation process is the *B. subtilis* mutant *ts-5* of Leighton et al. (181). This strain produced neither exoprotease nor antibiotic at the restrictive temperature and was blocked morphologically at stage 0 (262). The mutation mapped between *aroD* and *lys* but probably not in the *spo0A* locus (180). The mutation was in a locus whose expression was required from a time before the formation of extracellular protease until the appearance of refractile bodies, which was the last event examined (181). By a variety of criteria, the serine protease from the mutant was found to be less stable than the enzyme from the wild type. Moreover, the length of time required for expression of this locus is consistent with work using protease inhibitors, which indicated that the serine protease was required for at least 3 h during spore formation (55). Thus, there are grounds for thinking that the mutation might be in the structural gene for the serine protease but, as the authors themselves pointed out, it is possible that the differences between the serine proteases from the mutant and from the wild type could have resulted from some other primary lesion, the product of which might interact with the protease (180). In view of the uncertainty that exists over the role of the serine protease during sporulation, this qualification should be tested. Various mutations have been described (*cpsX, abs, tol;* see later section) that can overcome the defect in serine protease formation in a variety of *spo0* mutants (113, 150, 151). The effect, on serine protease production, of introducing one of these mutations into strain *ts-5* might resolve the point as to whether the lesion lies in the structural gene for this enzyme.

Szulmajster et al. (297) described a thermosensitive sporulation mutant (*ts-4*) that was also blocked at stage 0. It was unable to make antibiotic at the restrictive temperature, but was able to synthesize exoprotease and was therefore blocked later than *ts-5*. The mutation was linked to *ura* (294) and might lie in the *spo0E* locus. Temperature shift experiments indicated that the product of this locus was not needed for successful sporulation for as long as the product of the locus identified by the *ts-5* mutation. If the mutant was shifted from 42 to 30°C at any time up to 1 h after the end of growth, then sporulation was unimpaired. Similarly, a shift from 30 to 42°C after t_3, that is to say, more than 3 h after the start of spore formation, had a progressively decreasing effect on sporulation capacity. The wild type takes somewhat different times to sporulate at the two temperatures, making it difficult to assess, from the data given, a precise time for the requirement of the gene product. The level of intracellular protease activity was 15-fold lower in the mutant than in the wild type at the restrictive temperature (222). However, no firm conclusions could be drawn as to the function of the protein produced by the mutated locus.

For mutants *ts-4* and *ts-5*, spore formation was prevented by an initial incubation of more than about 1 h at the restrictive temperature in conditions that induce sporulation of the wild type. The fact that sporulation was not merely delayed by this treatment indicates that, at least in these two instances, the bacteria cannot wait for a "good" gene product to be pro-

duced. If the correct product is not present at the correct time, then the sporulation process is irreversibly altered and the possibility of forming spores lost. This indicates a rather delicate control of a sort not generally seen with metabolic pathways. If a general characteristic, it would explain the reported failure to obtain cross-feeding between sporulation mutants (196, 267). The irreversible loss of the ability to sporulate, or "commitment not to sporulate," is most clearly seen in experiments with a ts mutant (279.1) described by Young (335). This strain sporulated normally at 34°C, but at 42°C very few spores were formed and most of the cells were blocked at stage II, exhibiting an abortively disporic phenotype. The critical period was at about the start of stage II. At this time, as little as 15 min at the permissive temperature was all that was needed for the mutant to be able to sporulate. However, bacteria reaching the critical period at the nonpermissive temperature were almost immediately "committed not to sporulate." Experiments with inhibitors of ribonucleic acid (RNA) and protein synthesis indicated that the gene product required for the 15-min period was a protein coded for by a short-lived messenger RNA (mRNA). The mutation was located in the *spoIIG* locus (334). It therefore defines the point during sporulation, the length of time, and to some extent the nature, of the expression of at least one gene product of this locus.

Young (335) also characterized a thermosensitive strain (285.1) that sporulated to about 50% of the wild-type frequency at 32°C and was blocked at stages IV to V at 40°C. The mutation was linked to *cysA14* by transduction (M. Young, personal communication) and is tentatively placed in the *spoVB* locus. In this case, the temperature-sensitive period began at about the end of stage II, and the cells required 180 min at the permissive temperature in order to sporulate. From this, one might have predicted that the time at which the strain became temperature sensitive (temperature shift-down experiment) would have preceeded the time at which it ceased to be temperature sensitive (shift-up experiment) by a considerable proportion of the total time needed to form spores. However, the time at which the strain started to be temperature sensitive was very close to the time at which it finished being temperature sensitive (measured as a fraction of the total time needed to sporulate). The author suggested several possible explanations for this apparent paradox (335): that development could be arrested for some 200 min before cells are committed not to sporulate; that the mutated gene is expressed through a stable mRNA; that the mutated gene codes for, or controls, synthesis of a protein that begins to be made (about stage II) well before it is required.

Leighton (176) described a thermosensitive mutant, *ts-14*, that was shown to be blocked at stage II (before the formation of alkaline phosphatase) at the restrictive temperature, 47°C (263). The cell produced a single polar septum normally, but cell wall material was deposited in the septum membrane, and development proceeded no further. The start of the temperature-sensitive period preceeded the time of escape by about 30% of the total time taken for sporulation; this period covered approximately the middle third of the sporulation sequence. The defect was probably the result of a single mutation that also caused resistance to rifampin (25 out of 25 spo^+ revertants tested had regained sensitivity to rifampin). The mutation was closely linked to *cysA14* (80% cotransduction); this is compatible with a location in the *rif* locus, and hence in a structural gene for RNA polymerase. This was supported by the observation that RNA polymerase activity in vitro was resistant to rifampin. RNA synthesis by the mutant at the restrictive temperature continued, during the early stages of sporulation, at a much increased rate compared to the wild type, and Santo et al. (263) suggested that the RNA polymerse was altered in its transcriptional specificity so that it continued to transcribe a class of vegetative genes that were normally turned off in the wild type and were responsible for cross wall synthesis. A number of *rif* mutations have been described for *B. subtilis*, and some of these affect sporulation (see later section). All these mutations apparently map in the one locus. It would seem important to establish the proximity of the *ts-14* mutation of these other *rif* mutations (and to nearby *spoIIE* locus) by transformation crosses.

In an analagous study, Leighton (177) has described a thermosensitive strain, *ts-39*, that was blocked at stage 0 at the restrictive temperature and in addition resistant to streptomycin. As with the mutant *ts-14*, discussed above, the drug resistance was not temperature sensitive, and the strain exhibited the ts sporulation phenotype in the absence of the antibiotic. The sporulation and streptomycin resistance properties were consistently transferred together in genetic crosses, and a majority of Spo^+ revertants became streptomycin sensitive. This indicates that the two properties were probably the result of a single mutation. The 30S ribosomal subunit from *ts-39* had lost the ability to bind dihydrostreptomycin; it seems likely that the mutation lies in the *strA* locus and hence affects a protein in the 30S subunit. The mutant

was temperature sensitive virtually throughout sporulation, but not during vegetative growth. This might suggest that the mutation was specifically affecting the capacity of the ribosomes to translate sporulation mRNA. One can only speculate as to how the lesion might promote such temperature-dependent translational changes.

A number of other apparently sporulation-specific ts mutants have been mentioned in the literature, but detailed biochemical studies that take advantage of their thermosensitivity have not been reported. Rogolsky (246) reported five ts *B. subtilis* mutations that were linked to *ura;* these were not subjected to further analysis. Szulmajster and Keryer (301) described three mutants that had greatly reduced intracellular protease activity and exhibited ts protein turnover and spore formation; these traits resulted from more than one mutation, and consequently it is difficult to draw a satisfactory correlation between the different activities. Lundgren and Cooney (191) described three ts sporulation mutants of *B. cereus*; these were analyzed mainly with respect to metal ion uptake.

We have discussed the work on ts mutants in some detail because we believe that the potential of this approach is considerable. The few studies done so far have brought us nearer to identifying the role of certain *spo* loci. They have indicated that there is wide variation in the way that different loci are expressed. Some gene products seem to be required for only a short time at a specific point in the process, whereas others are apparently required practically throughout sporulation. There are too few studies at present to integrate this sort of information into a general scheme for the control of the expression of loci during sporulation. The main problem seems to be that very few mutants have been found that possess a suitably clear phenotype, sporulating well at the permissive temperature and poorly at the restrictive temperature. In addition, there is a problem of interpretation of results. The gene product, presumably a protein, may be temperature sensitive only during its synthesis and this may not coincide with the point at which the protein is required. Alternatively, the gene product may be temperature labile at all times and, in this case, the ts period coincides with the period during which the gene product is required. These possibilities complicate interpretation of results (see references 27, 155, 335). A large number of nonsense-like suppressible *spo* mutations are known (137), and it is possible that some of these problems could be overcome by the isolation of a temperature-sensitive suppressor mutant of *B. subtilis*. This would effectively make all nonsense mutations available for temperature shift studies, and the sensitive step would be at the point of synthesis. Such mutants are known in other species (27, 98, 236, 279), and should be obtainable in *B. subtilis*.

Putative Control Mutations

All the asporogenous mutations examined have been pleiotropic and therefore might be mutations in control elements. The mutants were normally identified by lack of pigment production (147, 269). This procedure might tend to select preferentially those mutants that are pleiotropic and hence possible control mutants. For example, mutants might be excluded that are unable to make one spore component, but otherwise produce nearly normal spores. In fact, an increasing number of mutants that produce normal numbers of modified spores have been identified by means other than pigment production. They include mutants that are unable to synthesize DPA (21, 337), mutants that produce lysozyme-sensitive spores or spore requiring lysozyme for germination, both of which have altered coat layers (6, 45), and mutants that overproduce sporulation-associated proteolytic activity (22). On the other hand, if the majority of sporulation events are part of a dependent sequence, then it would be expected that most sporulation mutations, including those in structural genes, would be pleiotropic. Whether these pleiotropic mutations should be looked on as control mutations per se or as mutations that indirectly affect the control of the sporulation process is, for the moment, a matter of semantics.

In the following sections, putative control mutations are considered whose characteristics could be explained by invoking some change in control mechanisms. For ease of presentation these may be described as control mutations, in contradistinction to asporogenous mutations. However, it should be borne in mind that some, at least, of the asporogenous mutations may also be mutations in control elements. Various types of putative control mutations are discussed in other sections and are not considered here; these include the different types of initiation mutation and the different types of mutation to drug resistance.

Oligosporogenous mutations. Mutations that prevent cells from forming spores can be divided phenotypically into two types. They may cause a completely asporogenous (Spo) phenotype, in which the mutants are incapable of producing any heat-resistant spores. Alternatively, the mutants may be oligosporogenous (Osp) and characterized by subnormal produc-

tion of heat-resistant spores under conditions that lead to normal sporulation of the wild type (269, 271). All degrees of sporulation may be found in Osp mutants from 1 spore in every 2 or 3 cells to 1 in 10^8 cells, but for any one mutant the degree of sporulation under defined conditions is constant. Spores derived from Osp mutants, when recultured, sporulate again at the same low frequency. This is the defining characteristic of the Osp phenotype. The obvious explanation for the Osp phenotype is that it results from a leaky mutation, where normal amounts of slightly active protein are produced. This could be in a structural gene or in a regulatory gene. However, it is also possible that the Osp phenotype results from expression of separate types of regulatory mutation, for example, promoter mutations. In either instance, the concept of a threshold level of some limiting factor is necessary to explain why only a fraction of a genetically homogeneous cell population are able to overcome the block. This would be reflected in physiological variation between cells in a culture. It is conceivable that the threshold level could be affected by the environment (especially if a general factor, such as ATP, is involved), and this may explain why some mutants have a variable incidence of spore formation when grown and allowed to sporulate with different carbon sources (8, 49).

It is clear that for most Osp mutants examined, the cells not going on to form mature spores are blocked at defined stages in the morphological sequence of spore formation, and exhibit many of the features of Spo mutants (23, 69, 257). Coote (50) found that this was so for most of a group of 30 Osp mutants, and that the majority of the mutations mapped in areas of the genome previously shown to be occupied by *spo* mutations. In particular, many mutations were located in the *phe-lys* segment in positions that corresponded to *spo* mutations of similar phenotype. It seemed reasonable to suppose that the Osp and Spo phenotypes were alternative expressions of mutations within a single gene. The possibility that the Osp state still reflected a qualitative, rather than a quantitative, distinction from the Spo state could be discounted if both types of mutation were shown to be located in a single gene. In a number of instances, RIs from transformation crosses have indicated that the two types of mutation were indeed located in the same gene. Rouyard et al. obtained an RI in the range 0 to 0.03 in reciprocal crosses between an Osp and two Spo stage II mutants (250). Coote reported an RI low enough (<0.1) in reciprocal crosses between one Osp and two Spo mutants to suggest that all the mutations were located in the same gene within the *spoIVC* locus (50). No recombination was detected beween two mutations in the *spoOH* locus, such that one resulted in a Spo and the other an Osp phenotype (227). For both the *spoVC* (143) and *spoIVA* (P. J. Piggot, unpublished observation) loci, RIs in crosses between an Osp and a Spo mutant have also been low enough to suggest that the two types of mutation would lie in the same gene within the locus.

Double Osp mutants have been constructed in an attempt to obtain further information on the nature of oligosporogeny (52). This was done to investigate the possibility that the small proportion of cells able to sporulate in the population resulted from some general factor that could enable cells to overcome more than one type of oligosporogenous mutation. In this case, the amount of the hypothetical general factor would be determined by the more severe lesion, and this amount would be adequate to overcome both lesions. Thus, if the two mutations taken singly allowed, for example, 1 and 10% sporulation, then the double mutant would show 1% sporulation. If, however, the factors involved were entirely independent, then the probability of sporulation in the double mutant would be the product of the separate probabilities, or 0.1%. In the double mutants examined, the latter situation was found, indicating that independent factors were involved in overcoming the different oligosporogenous blocks studied (52).

The evidence discussed above favors the interpretation that Osp mutants have leaky mutations, and that Spo and Osp phenotypes can arise from different mutations in the same gene. This does not rule out the possibility that some Osp strains may have a distinct type of mutation in a regulatory function, and there are several cases where this may be so. First, distinct Osp phenotypes with no Spo counterpart have been described (23, 49), although it could be argued that this merely reflects the fact that too few Spo mutants have been examined. Second, a number of Osp mutants were reported (22, 23) that apparently had increasing difficulty in overcoming each stage in the process, so that only a few cells formed spores. Even 20 h after initiation of spore formation, cells revealed all the stages of sporulation, with a preponderance of early stages. It was suggested that this phenotype might result from a mutation in a distinct type of general regulatory mechanism. However, no representative of this type of mutant was found in a later study of Osp mutants (49), and it is possible that these strains may have been double mutants, or mutants damaged in some vegetative function.

Third, all the mutations so far placed in the *spo0E* locus give rise to an Osp phenotype (50, 136, 294; see description of locus). Where several mutations have been located in other loci, these have generally given rise to Spo as well as Osp phenotypes. Thus there is a possibility that the *spo0E* locus is of a distinct regulatory type (37).

In summary, it is clear that most of the evidence is strongly in favor of the supposition that the Osp and Spo phenotypes are alternative expressions of mutations within the same gene, although it is still possible that some Osp mutants may result from mutations within distinct regulatory mechanisms.

Mutations that alter the timing of events. Fitz-James and Young (82) described a cortexless mutant of *B. cereus* that laid down spore coat layers 1 h earlier than did the wild type. Later events, such as DPA synthesis and Ca^{2+} uptake, occurred normally in the mutant. It would be interesting to know whether the altered timing was caused by premature coat deposition, preventing cortex synthesis, or by failure to synthesize cortex, allowing coat deposition to take place earlier. Later work suggested that the latter interpretation was correct (224); although as no Spo^+ revertants were found (82), the two effects could have resulted from different mutations.

A class of mutants ("late abnormal derepressed," or Lad) has been described in which spore development is delayed (21, 22). This type of mutant was originally isolated as an overproducer of elastase activity (19). Spore development in these strains proceeded normally up to about stage III, at which point it was delayed for several hours before eventually continuing to give a high yield of heat-resistant spores. After sporulation was completed, the sporangia contained inclusions of material similar in appearance to the spore coat, and the spores themselves had abnormally thick coat layers (283, 330). It was suggested that this morphology resulted from continued production of the coat proteins during the "idling," or delay, period. The cessation of protease and alkaline phosphatase production that occurs at about stage III in the wild type was not observed in the Lad mutants, and this resulted in overproduction of these sporulation-associated functions. It was suggested that the inability to switch off, at the correct time, certain functions normally active up to stage III caused their overproduction, and this retarded subsequent stages. The complex phenotype was reported to result from a single mutation (21), and it will be interesting to see how mutations of this type map in relation to the known *spo* loci.

The Lad mutants are distinguished by virtue of their ability to "idle" for several hours before completing the process of spore formation. As discussed earlier, studies of ts sporulation mutants blocked at stages 0 or II had indicated that these strains were rapidly committed not to sporulate if incubated for any length of time at the restrictive temperature (181, 297, 335). However, one interpretation of the behavior of a mutant blocked at stage IV to V at the restrictive temperature was that development could be arrested for some 200 min before there was irreversible loss of the ability to sporulate (335). It may be that "idling" is possible after completion of the forespore (stage III). One can but speculate as to whether this has anything to do with the commitment to sporulate that may also be associated with this stage (see later section).

Bypass mutations. Asporogenous mutations are highly pleiotropic, causing the loss of a number of characters associated with sporulation of the wild type. This has made it possible to isolate partial revertants of *spo* mutants that have regained some, but not all of the wild-type characters. These can be distinguished from full revertants and nonsense suppressor mutants. The partial revertants retain the original *spo* mutation and remain asporogenous, but a bypass mutation has somehow disrupted the dependent sequence of sporulation events so as to allow the expression of certain sporulation-associated events, despite the *spo* mutation. A study of the epistatic relationship of such bypass mutations with *spo* mutations might be expected to improve our understanding of the way in which *spo* loci are expressed, and of how that expression is organized. So far, the studies have been perplexing rather than illuminating. They add to the number of sporulation-related loci whose expression is not understood. In no case have the bypass mutations been found to interfere with the sporulation of spo^+ strains.

Most of the work has concerned a series of markers that are associated with stage 0 mutants and are thought to indicate membrane alterations (112, 113, 150, 151). Both Guespin-Michel (112, 113) and Ito et al. (150, 151) isolated several distinct types of bypass mutant. For several of these, they made use of the fact that certain stage 0 mutants are more sensitive to polymyxin and to the antibiotic produced by the sporulating wild type than are later-blocked mutants or the wild type. The bypass mutations, called *cpsX* (113), *absA*, *absB*, *absC*, *absD* (151), *tolA*, and *tolB* (150), caused the asporogenous mutant to regain sporulation-associated functions to varying degrees. For some bypass mutants, these included formation

of antibiotic and extracellular protease. As these were expressed in strains harboring *spo0A* (Pr^- Ab^- type), *spo0B*, or *spo0F* mutations, it established that these *spo* mutations could not be in the structural genes for the exoprotease or the enzymes involved in antibiotic synthesis. The map locations for these various bypass mutations are not known, but they are clearly distinct from the *spo0A* and *sop0B* loci. The *tol* mutations were placed close to *trpC* in a recent review (332), but this is not compatible with the published data (150) and would seem to be an error.

Guespin-Michel reported a detailed analysis of the *cpsX* mutations (113). These were closely linked to each other in transformation crosses and so were considered to lie in a single locus. However, different *cpsX* mutations gave rise to five distinct phenotypes in a *spo0A* mutant, and the phenotype resulting from a particular *cpsX* mutation depended on the *spo0* mutation (113). How such a range of phenotypes could result from mutations at a single locus is difficult to envisage. Guespin-Michel suggested that the locus could code for a membrane protein, and it is also possible that *cpsX* is a complex regulatory locus. Any final explanation will have to account for the different phenotypes in precise molecular terms.

Most of the parameters used in these studies are not particularly amenable to quantitative assays, and there are no reports of the kinetics of appearance of the various stage 0 markers in the bypass mutants. In an attempt to overcome this difficulty, Piggot and Taylor (230) used as a marker the ability of the wild type to synthesize alkaline phosphatase during sporulation in the presence of inorganic phosphate. This is associated with stage II and is lost by mutation in any *spo0* locus or in certain *spoII* loci (Table 3). Thus, its appearance is closely related to the dependent sequence of sporulation events. Two loci, *sapA* and *sapB*, were described, mutation in which enabled the *spo* mutants tested (with mutations in the *spo0K*, *spoIIA* and *spoIIE* loci) to form the sporulation alkaline phosphatase. It seems likely that the *sap* mutations were in control elements, such that formation of the enzyme was no longer dependent on the correct expression of the *spo* loci. However, the bypass mutants only formed the enzyme under sporulating conditions, indicating that its synthesis was still under some form of sporulation control. The *sapA* and *sapB* mutations mapped near to *metC* and *purB*, respectively, and were distant from *phoP*, *phoR* (174, 207) and the *spo* loci.

The nature of the bypass mutations is unknown. They are phenotypically and, where it has been tested, genetically distinct from mutations in *spo* loci. Consequently, they add to the number of loci whose expression may be involved in the formation of an endospore.

Analysis of Merodiploids

A merodiploid system in *B. subtilis* has recently been described by Audit and Anagnostopoulos (14, 15). The merodiploid condition was observed after transformation or transduction to prototrophy of strains bearing the *trpE26* mutation. This mutation has its origin in *B. subtilis* 166, and strains carrying the *trpE26* mutation apparently have a major rearrangement (translocation) of part of the chromosome, compared with *B. subtilis* 168 (314). Prototrophic transformants or transductants of *trpE26* were unstable *trp*$^+$/*trpE26* heterogenotes (segregating *trpE26* progeny) and diploid for a considerable portion of the chromosome. This system has been used to construct *spo*/*spo*$^+$ heterogenotes to study the dominance relationship of certain *spo* mutations (157, 158; D. A. Broadbent, personal communication). The analysis is time-consuming and consequently, few mutations have been studied. In general, DNA or a transducing lysate of the *spo* mutant was used to convert the *spo*$^+$*trpE26* strain to prototrophy. Each merozygote clone was purified by a single colony isolation, grown in broth, and plated to determine the pattern of segregation of the haploid markers. In several cases analysis was confirmed by heat treating the merozygote clone and studying the outgrowth and segregation pattern of the surviving spores. In a number of instances the results were consistent with the *spo* mutation being dominant to the *spo*$^+$ allele (Table 5). The apparently dominant *spo* mutations tempt speculation that they are mutations in control genes, but it is also possible that the dominance results from aggregation of a mixture of functional and nonfunctional subunits of a structural protein. The latter interpretation seems particularly likely for a mutation in the *spoIVA* locus that caused deposition of spore coat material in the sporangium in a similar manner to the mutation in strain P20 (Fig. 5) that is in the same locus. The interpretation of diploid analysis is difficult even in well-developed systems (29), and it would seem premature to attempt any far-reaching analysis relative to sporulation. The situation with respect to the *spo0A* locus was discussed in the section on initiation mutants.

Dominance of a *spo* mutation over the wild-type allele confounds complementation analysis, and there are no published reports of successful analyses with recessive mutations. This

TABLE 5. *Dominance relationship of various spo mutations to the wild type as derived from studies of merodiploids constructed by virtue of the trp⁺/trp E26 heterozygosity*

Locus	Mutant	Type	Reference
spo0A	3NA, 5NA, 6U	Dominant	157
spo0A	NG6.21	Dominant	D.A. Broadbent, personal communication
spo0A	9V	Recessive	158
spo0B	6Z	Recessive	158
spoIIA	4Z, 4SA	Recessive	158
spoIIIA	7Z	Recessive	158
spoIIIA	NG17.17	Recessive	D.A. Broadbent, personal communication
spoIVA	NG17.23	Dominant	D.A. Broadbent, personal communication
spoIVC	E13	Dominant	D.A. Broadbent, personal communication

would be particularly interesting for the *spoIIIA* locus where a number of mutations are known (Table 1) and where there is suggestive evidence for several genes (227). However, if two linked *spo* mutations are shown to complement each other, it may prove difficult to distinguish between inter- and intracistronic complementation, since the immediate gene products are not being assayed quantitatively.

Commitment

Sporulation is initiated by starvation conditions, and sporulating bacteria are said to become committed to endospore formation when they can complete the process even when starvation is relieved and growth conditions are restored. This is the sense in which the term "commitment" is generally used, and it is the sense used here, although Balassa (20) has preferred the term "irreversibility" to identify this phenomenon and used "commitment" to denote the induction of sporulation-specific events.

The phenomenon of commitment was noted many years ago (26). As judged by ability to form refractile spores, commitment with respect to glucose addition was found to occur at a fairly precise time in the process (110, 123), and under certain conditions can occur very soon after initiation (90). This early point of commitment with respect to glucose addition was found to coincide with a reduction of the ability of cells to take up glucose (90). Thus, commitment could be regarded as a reflection of altered permeability properties (90, 94). This explanation implies that sporulation loci remain susceptible to repression by catabolites, at least until the time of commitment. The point of commitment with respect to addition of fresh broth to sporulating cells has generally been found to be at the stage of protoplast formation (stage III: 81, 95, 108, 128). It is clear that the precise point at which commitment occurs depends on the medium in which the cells are sporulating and on the enriching medium used (94). Yet in a variety of experimental systems, the latest point of commitment has coincided with the formation of the protoplast free within the mother cell cytoplasm. At this time, the opposite orientation of the outer prespore membrane with respect to the cytoplasmic membrane (see [325]) probably hinders passage of enriching nutrients into the prespore, although not the mother cell. Thus, it would still be possible to explain commitment in terms of altered permeability of the prespore.

Altered permeability properties may not be a sufficient explanation for all types of commitment. In particular, Sterlini and Mandelstam (291) examined the commitment of *B. subtilis* to the production of various sporulation-associated functions when hydrolyzed casein was used to enrich cultures undergoing sporulation in a minimal replacement medium. They found no single point of commitment for the functions they examined; cells were committed to form alkaline phosphatase before they were committed to form refractile spores, and they were committed to refractility before they were committed to synthesise DPA. This successive commitment to one event after another is difficult to explain on the basis of permeability, and implies some additional mechanism. For example, the temporal sequence of gene activation during spore formation may be susceptible to repression by added nutrients at a number of points. It will be interesting to see whether this succession of commitment points is obtained with respect to the addition of a single component rather than a complex mixture such as casein hydrolysate.

Up to the point of commitment, it is clear that enrichment of *B. cereus* causes synthesis of cell wall material in the spore septum, and both the sporangium and prespore compartment are able to resume vegetative growth (81). With *B. subtilis* at stage II only the sporangium was reported to be able to grow out as a vegetative cell (95); the molecular mechanism for this is not known.

Mutations to Antibiotic Resistance That Also Affect Sporulation

The *ts-14* and *ts-39* mutants (176, 177) discussed in a previous section are two examples of

a growing collection of mutants that are drug resistant and concomitantly altered in their sporulation pattern. Most involve mutations that are known to alter either the ribosome or RNA polymerase, and they represent a minority of all such mutations to drug resistance, the majority of which do not affect sporulation. In a number of cases, the complex phenotype has been shown to result from a single mutation. The mutations have the great advantage, over many other types of sporulation mutations, that the primary genetic lesion can be defined. This does not mean that the precise manner in which they affect sporulation is defined, but it does mean that the mutations may provide some evidence about the process.

Mutations that affect transcription. Rifampin (Rif), streptolydigin (Std), and streptovaricin are known to affect RNA polymerase (323), and mutants resistant to these antibiotics have been described in *B. subtilis*. The mutations are located in a small cluster between the *cysA* and *strA* loci (124, 127, 280). Doi and collaborators described a Rif^r mutant (DB-31) that produced odd-shaped spores, heterogeneous in size, and often longer and narrower than the wild type (65, 165). Sonenshein and Losick (281) described a mutant (*rfm-10*) that was unable to sporulate, and has since been shown to be blocked at stage 0 (280). A Rif^r mutant that was temperature sensitive for sporulation and blocked at stage II at the restrictive temperature (176, 263) was discussed in a preceding section. In all cases, the RNA polymerase was shown to be altered in its sensitivity to rifampin, and there was strong evidence that the mutant phenotype was caused by a single mutation.

Sonenshein et al. (280) carried out a detailed study of a series of Rif^r and Std^r mutants of *B. subtilis*. They found that the majority sporulated as well as the parent strain, and identified three other types that were altered in their ability to sporulate: (i) mutants that were asporogenous, whether or not the drug was present; (ii) mutants that were drug resistant during growth, but whose sporulation capacity was markedly drug sensitive (these accounted for no more than 5% of the resistant mutants isolated); and (iii) a rare class that was rifampin sensitive during growth and resistant during sporulation. All the asporogenous mutants that were characterized by electron microscopy were blocked at stage 0; they produced exoprotease and antibiotic. A subclass of the asporogenous mutants was temperature sensitive for sporulation. Transformation crosses established that the mutations to rifampin and streptolydigin resistance were located between *cysA14* and *strA1* in closely linked, but nonoverlapping, loci.

Loss of the ability of RNA polymerase to transcribe DNA of the lytic bacteriophage ϕe in vitro is an early event in sporulation (40, 189) and has been taken to indicate a change in template specificity of the enzyme at the onset of sporulation. This change did not occur in Rif^r mutants blocked at stage 0 of sporulation (280, 281), with the exception of one mutant examined by Sonenshein et al. (280). Purification of RNA polymerase had indicated that the mutation to rifampin resistance alters the β subunit, which is in the core of the enzyme (38, 186, 187). Precisely how the alteration affects sporulation has not yet been established. It has been suggested that the enzyme can no longer recognize sporulation promoter sites because of a lack of interaction with sporulation-associated proteins equivalent to the vegetative σ factor (109, 185). Many reports have indicated the presence of more than one type of RNA polymerase in sporulating cells (38, 63, 96, 126, 163, 185, 218), and this is consistent with the idea that polymerase structural changes, or changes of associated protein factors, play a role in controlling the transcription of spore genes. The presence of more than one RNA polymerase is not surprising, since it is clear that sporulating cells continue to make vegetative mRNA (2, 64, 66, 292, 328).

The evidence is clear that mutations to Rif^r or Std^r lead to an altered RNA polymerase, and apparently as a consequence there is a possibility that the mutants will be defective in spore formation. However, precisely how this alteration affects sporulation has yet to be established, and a note of caution should be introduced into the interpretation of these various results. First, there is some doubt that the change in specificity of RNA polymerase with respect to ϕe DNA as template has any relevance to sporulation. Murray et al. (214) found little change in specificity with respect to ϕe DNA when the enzyme was extracted from cells induced to sporulate in a resuspension medium, although they confirmed the observation (189) that the specificity changed when bacteria were induced to sporulate by exhaustion in a broth medium. Similarly, Orrego et al. (222) suggested that the change of polymerase specificity was quantitative rather than qualitative, and that the template specificity changes measured with templates other than *B. subtilis* DNA may merely reflect physiological differences unconnected with sporulation. Second, it has been reported that the inability of a Rif^r mutant to sporulate could be corrected by the addition of arginine, methionine, valine, and isoleucine to

the sporulation medium (235). The mutation was 25% cotransformed with *cysA14*, and in transformation crosses, the Rif^r trait was inseparable from the loss of ability to sporulate. In this case, it would seem unlikely that the ability to sporulate under certain conditions resulted from an inability of the altered polymerase to transcribe sporulation genes. It would seem more likely that the altered transcriptional activity promoted a requirement for exogenous amino acids because, perhaps, the genes for amino acid synthesis were not efficiently transcribed in the mutant. The amino acids might have no obvious effect on growth, but have a pronounced effect on spore formation where they may be required in greater amounts (89). Whatever the explanation, the suggestion is strong that asporogeny resulting from an alteration in RNA polymerase is an indirect consequence of the mutation, and is not caused directly by an altered specificity toward sporulation genes. Third, as noted above, a class of mutants was obtained (280) that was rifampin resistant during growth, but whose sporulation was suppressed by exposure to the drug. This phenotype is difficult to explain, as the authors point out, other than by suggesting that sporulating cells are more permeable to the drug, or that binding of the drug to the RNA polymerase is somehow enhanced when the enzyme is active at sporulation promoters. The authors also described another type of mutant that was sensitive to rifampin during growth, but resistant during sporulation; this phenotype could adequately be explained by reasoning opposite to the above, and so it does not seem valid to use the existence of either type of mutant as evidence for a specific role of RNA polymerase in sporulation. In view of the damaging effects of rifampin on *B. subtilis* (53), caution is required in the interpretation of any experiments requiring the presence of the antibiotic.

A mutant of *B. subtilis* has been reported that is resistant to the DNA-intercalating agent ethidium bromide (32). It was also resistant to acriflavine and proflavine, but the mutation was linked to *hisA1* (32) and so was distinct from the mutation to acriflavine resistance described by Ionesco et al., which mapped between *phe* and *lys* (148). The mutant was resistant to ethidium bromide during growth, but only formed spores in the absence of the drug. The mechanism of resistance is unknown, but such drugs are of potential value in studies of spore formation as Sakaran and Pogell (261) have reported a differential inhibition of catabolite-sensitive enzyme induction in enterobacteria by intercalating agents. In this respect, Rogolsky and Nakamura have reported that sporulating cultures of *B. subtilis* wild type are particularly sensitive to ethidium bromide after the formation of the serine protease, but before septation (247). This may indicate an enhanced sensitivity of certain sporulation genes.

Mutations that affect translation. A wide range of antibiotics interact with the bacterial ribosome (324). Drug-resistant mutants are known in which the resistance results from an altered ribosome, and many such mutants have been described for *B. subtilis* (104, 161, 223, 278, 308). Alteration of the ribosome could conceivably interfere preferentially with the translation of sporulation-specific mRNA, and there has been a search for ribosomal mutants that are altered in their ability to sporulate. However, resistance to an aminoglycoside antibiotic does not in itself establish that a mutation is ribosomal. Indeed, a number of aminoglycoside antibiotic-resistant mutants that are altered in spore formation result from mutations that are probably not ribosomal (162, 202, 287, 303). For this reason, authors have been cautious in their comments about other drug-resistant asporogenous mutants in which the lesion could be ribosomal (106, 177).

There are two reports where these doubts have been overcome. The first concerned the Str^r *ts-39* mutant of Leighton (177), and this was discussed in a previous section. The second, by Domoto et al., described erythromycin-resistant mutants of *B. subtilis* that did appear to have ribosomal mutations and that were altered in their sporulation phenotype (67). The strains were resistant during growth, asporogenous in the presence of the antibiotic, and able to sporulate normally in its absence. The conditional asporogenic phenotype was cotransformed with erythromycin resistance in all cases. The mutations mapped in the region previously shown to contain the ribosomal erythromycin resistance, *ery*, locus (104, 124). The antibiotic-sensitive period extended for the first 5 h of sporulation. Evidence was obtained that one of the 50S ribosomal proteins was altered in the mutants. As the authors pointed out, this does not explain why ribosomes from the mutants bound erythromycin as efficiently as those from the wild type, nor why the strains were asporogenous in the presence of the drug.

Fortnagel and Bergman reported the isolation of several hundred fusidic acid-resistant mutants of *B. subtilis* that were oligosporogenous (83). Resistance to this antibiotic is associated with elongation factor G (105), and the authors suggested that alteration of the G-factor reaction might prevent some alteration in

translational machinery necessary for sporulation.

There are, then, several cases where antibiotic-resistant mutations have been shown to alter the translational machinery and also to affect sporulation. However, this still does not establish a direct effect on spore formation of the alteration in the translational machinery. The demonstration of such a direct effect must await in vitro studies in which translation of spore-specific mRNA is shown to be specifically increased by alteration of the ribosomes or ribosome-associated factors.

Perhaps, too critical an attitude has been adopted in the discussion of drug-resistant mutants, whether they affect transcription or translation. If so, this is because it is possible to draw far-reaching, and as yet unsubstantiated, conclusions about the role of certain transcriptional and translational changes in the control of spore formation. It is also because there is, for once, some solid data about the possible types of control. It is worth considering what might have happened if rifampin, for example, had not been discovered. Asporogenous rifampin-resistant mutants could not have been isolated as such. However, the same mutants might have been isolated as stage 0 sporulation mutants (it is surprising that none has been found in this way). It would probably have been assumed that the mutations were in the, supposedly sporulation specific, *spo0H* locus that is closely linked to *rif* by transformation (227). (One could speculate further and ask whether some of the *spo0H* mutations already mapped cause an alteration in the RNA polymerase.) The mutations would thus have been reduced to the level of blissful ignorance that characterizes our knowledge of the immediate biochemical consequences of *spo* mutations.

Molecular Mechanisms for Regulation of Sporulation Events

Evidence discussed in the preceeding sections has indicated that the changes which occur after initiation of spore formation are primarily controlled by sequential transcription of the relevant loci. There is also evidence to suggest that supplementary controls may operate on the translation of mRNA and on the post-translational modification of proteins. In this section, the types of control that might operate are briefly considered.

Transcriptional control. DNA-RNA hybridization competition experiments in several laboratories have indicated that sporulating bacteria produce new species of mRNA (2, 64, 66, 226, 255, 292, 328). This provides a prima facie case for transcriptional control. Reports of sporulation-specific DNA-binding proteins (35, 36, 262; H. A. Foster, personal communication) are consistent with this. The DNA-RNA hybridization experiments also establish that vegetative mRNA continues to be synthesized, and indeed it is known that many vegetative functions continue to be expressed (166), during spore formation. Further, several catabolic enzymes, β-galactosidase (9), histidase (51, 291), sucrase (51), and α-glucosidase (51), are inducible during sporulation, so that the capacity to synthesize new vegetative functions is not lost; a point emphasized by the commitment experiments. Any mechanism proposed for the transcriptional control of spore formation must allow for this continued ability to express vegetative functions.

Nearly all the work on molecular mechanisms of transcriptional control has concentrated on the possible role of RNA polymerase. An analogy has been sought between sporulation and the events that accompany bacteriophage infection of a host bacterium (see [42, 188, 294]). Two main lines of research have been followed. The first is the use of Rif^r (or similar) mutants, and this has established that RNA polymerase is necessary for sporulation, but has not established a specific role (see previous section). The second is the isolation and characterization of RNA polymerase and its associated factors, and the attempt to relate changes in these to changes in enzyme specificity during spore formation. Contrary to earlier reports, recent studies have shown that core RNA polymerase from sporulating cells is the same as the core enzyme from vegetative cells (186, 222). Recent reports indicate that there is interference of the binding of vegetative σ factor to RNA polymerase during sporulation, although the σ factor itself does not disappear (71, 311). Consequently, it is now thought that modification of RNA polymerase specificity may occur through interaction with other protein factors. Thus, much attention has focused on the isolation of protein factors that appear during sporulation and are able to bind to the RNA polymerase core (39, 96, 109, 138, 163). As yet, none of these proteins has been shown to modify the specificity of RNA polymerase.

It should be noted that the claimed change in template specificity of RNA polymerase during sporulation is usually based on a decrease in the ability of the spore enzyme to transcribe ϕe DNA in vitro. This observation remains controversial, and Szulmajster (295) has suggested that this change in template specificity is a characteristic of stationary-phase RNA polymerase rather than a reflection of sporulation-specific changes (see also 160, 214, 309). Before

changes in specificity of the enzyme can be considered to be established as a significant control mechanism, it must be shown that the spore enzyme is able to synthesize RNA transcripts in vitro that are not produced by the vegetative enzyme. However, a change in RNA polymerase specificity remains an attractive candidate for a control mechanism. It is consistent with the presence of more than one type of RNA polymerase during sporulation, which is presumably a reflection of the different proteins bound, as the core enzyme remains unchanged. The large number of *spo* loci are probably transcribed at a number of different times, and this makes it seem unlikely that the specificity of the RNA polymerase could be the only controlling mechanism. This would require a series of different modifying proteins to be synthesized at numerous stages during sporulation. It is not obvious how this would account for the dependent nature of the sequence of gene expression (see [320]), for which sequential induction remains a more attractive explanation.

Comparatively little attention has been given to other possible general types of transcriptional control. It has been known for some time that ultraviolet irradiation of spores causes the production of an unusual thymine photoproduct instead of the normal thymine dimer (68, 288). Irradiation of sporulating cultures of *B. cereus* at stage IV or later promoted formation of this "spore photoproduct" (17). This coincided with a change in the appearance of the chromatin of the prespore in electron micrographs. It was suggested that this represented a progressive change in DNA conformation (from B to A helical form), which favored formation of "spore photoproduct" upon irradiation. The change in DNA conformation would have a significant effect on transcription (17, 68). How the change in conformation is brought about and maintained is not clear, although it was closely paralleled by Ca^{2+} uptake (17). Other plausible candidates for this role are the two low-molecular-weight proteins which constitute about 15% of the protein in the spore core and which are rapidly degraded during germination (274). These proteins are not present in growing cells and appear in prespores at about the time of the change in the DNA. In addition, they can bind to DNA and alter its melting temperature by as much as 20°C (275).

Strong evidence for a change in DNA structure playing a central role in eukaryotic differentiation has come from studies with 5-bromo-2'-deoxyuridine (BUdR), an analogue of thymidine. Incorporation of this compound into the DNA of eukaryotic cells can suppress aspects of cellular differentiation and embryogenesis without affecting cell growth or gross RNA and protein synthesis (141, 252). Although other explanations are possible (see [139, 252]), it is likely that the incorporation of BUdR into DNA selectively modifies transcription (130), and current evidence suggests that this occurs by affecting the binding of regulatory proteins to DNA (172). This may also be true for prokaryotes as purified *lac* repressor from *E. coli* was found to have a substantially higher affinity for BUdR-substituted *lac* operator DNA (184). Preliminary experiments have indicated that incorporation of BUdR into the DNA of a thymidine-requiring, BUdR-tolerant strain of *B. subtilis*, at a level that did not affect growth, effectively prevented spore formation (J. G. Coote, paper presented at the British Spore Group Meeting, Leeds, 1975). In the presence of BUdR, the level of refractile spores was about 1% of that given by control cultures containing thymidine. Examination of the bacteria by electron microscopy indicated that BUdR incorporation had prevented most of the cells reaching the stage of spore septum formation. This effect of BUdR incorporation into DNA on the sporulation of *B. subtilis* seems comparable to the specific inhibition of eukaryotic differentiation by this analogue. Interestingly, exoprotease and alkaline phosphatase activities in the presence of BUdR were markedly increased over the control values. This overproduction is reminiscent of that seen in certain *spo* mutants (B. N. Dancer, personal communication) and in the *lad* mutants (22). The increase in alkaline phosphatase activity is normally associated with stage II, but here the increase occurred without the accompanying morphological changes.

Modification and restriction of DNA have been postulated as control mechanisms in developmental systems (139, 260). For example, enzymic modification by base methylation of an operator or promoter sequence might prevent repressor binding or enhance RNA polymerase binding and so allow transcription. Restriction and modification systems are known in *B. subtilis* (7, 276, 313). The inability of bacteriophage $\phi 2$ to replicate biologically active DNA in wild-type *B. subtilis* (152) might result from the activity of a host restriction enzyme, since the DNA made is smaller in size than that isolated from permissive hosts. Bacteriophage production occurs normally in *spo0A* and *spo0B* mutants (152), suggesting the possibility that these mutants are unable to make the restriction enzyme. Certainly, it is known that considerable amounts of DNA are excreted at the onset of sporulation (13, 79). Whether this is the result of the action of restriction enzymes, and

whether it has any functional significance is not known. The suggestion that restriction or modification systems might be involved in spore formation is, at present, only speculative.

Transcriptional control implies the regulation of specific sporulation operons by regulatory proteins, but as yet there is no direct evidence for this. However, critical experiments may soon be possible. Plasmids have recently been described for *B. subtilis* (190), and these, coupled with the rapid advances in restriction endonuclease techniques, may make it possible to insert specific sporulation operons into independently replicating plasmids as a prelude to a study of their control in vitro. Similarly, the recent report of condensed nucleoids from *B. subtilis* that can be transcribed in vitro (L. Hirschbein et al., paper presented at the British Spore Group Meeting, Leeds, 1975) may make it possible to test in vitro the more general types of transcriptional control mechanisms.

Translational control. There is now an increasing body of evidence to suggest some degree of control over sporulation at the level of translation. This evidence falls into three main categories: genetic or phenotypic modification of the components of the translational machinery; changes in translational specificity of in vitro protein synthesizing systems; synthesis of stable mRNA during sporulation.

Mutants that are resistant to antibiotics known to inhibit ribosome function, and that are concomitantly sporulation deficient, have been discussed in a previous section. These studies have offered no direct evidence that a ribosomal alteration is a functional requirement for sporulation. Similarly, differences in ribosomal composition (85, 164), changes in the levels of transfer RNA (tRNA) species (319) and modifications in the behavior of tRNA synthetases (290) need not be directly related to the control of sporulation. They could occur as subsidiary responses to the starvation conditions promoting sporulation or, since little or no translation takes place in the dormant spore (164), they may be preparatory events for dormancy rather than precise control mechanisms.

To establish the existence of translational control it is necessary to demonstrate a change in the specificity of translation. Thus far, there are two sets of in vitro experiments suggesting there is such a change. First, the "idling" reaction of vegetative ribosomes produced the nucleotides MS1 and MS2, whereas ribosomes from sporulating bacteria produced HPNI and HPNII (241; see earlier sections). Their production may reflect a functional change in translation during sporulation. Second, a fully active in vitro protein-synthesizing system from *B. subtilis* capable of translating exogenously added natural mRNA has been described (46, 173). Using this system it was shown that the ability of NH_4Cl-washed ribosomes to translate bacteriophage SPO-1 RNA in the presence of an initiation factor (IF) fraction from sporulating cells was greatly reduced, compared with the ability of the same ribosomes to translate SPO-1 RNA in the presence of an IF fraction prepared from vegetative cells. This difference did not extend to other RNA templates, so that there was a specific change in template specificity rather than a general loss in activity. Moreover, the IF fraction from a stage 0 mutant did not show this discrimination against SPO-1 RNA, so that the change appeared to be a sporulation-associated event. However, until such sporulation extracts can be shown to discriminate positively for sporulation-specific mRNA, the role of this template specificity change in the control of sporulation must remain suggestive rather than proven.

The stability of mRNA during sporulation has important implications for the extent of translational control. Unfortunately, conflicting evidence has been obtained on this point. Some studies have suggested the existence of stable mRNA (2, 4, 291), whereas others have suggested unstable mRNA for sporulation events (18, 81, 178, 179, 298, 299, 312). Intermediate values of mRNA stabilities (half-life about 10 min) have been reported in two specific instances: for dihydrodipicolinate synthetase in *B. subtilis* (47) and for the crystalline parasporal protein of *Bacillus thuringiensis* (100). Most of these studies have used either actinomycin D or rifampin to block RNA synthesis, and the general point of difference has been the concentration of antibiotic used. The studies that suggested stable mRNA used relatively low concentrations, which may have been insufficient to inhibit all mRNA synthesis. Conversely, studies suggesting unstable mRNA used relatively high concentrations, which are known to cause side effects (53). There have been two attempts to avoid this dilemma by the use of mutants. In the first, Leighton and Doi (179) used a Rif^r mutant that was apparently temperature sensitive for RNA synthesis. Their results indicated unstable mRNA for a number of sporulation-associated functions, but it was not established unequivocally that the synthesis of RNA was the temperature-sensitive step. In a later study, Leighton (178) used a mutant that was resistant to low concentrations of rifampin and sensitive to high concentrations. Addition of rifampin prevented sporulation. When rifampin was re-

moved, the strain sporulated normally, so that in this case the drug did not appear to have a lasting effect. These results also indicated short-lived mRNA.

Post-translational control. Post-translational control could presumably operate by influencing the assembly of subunits into some larger structure. Formation of the spore coat could well fall into this category. Coat material only becomes visible in electron micrographs at stage V, yet pulse-chase experiments have indicated that its synthesis starts very much earlier (5). Using antisera raised against alkali-soluble coat protein, Wood (327) confirmed this observation and, in addition, demonstrated the presence of cross-reacting material in *spo* mutants blocked as early as stage II. These strains never developed coat material that could be recognized in electron micrographs. This, together with evidence obtained from studies of double mutants (see earlier section), suggests a specific mechanism operating to control the assembly of accumulated coat proteins into the final coat layers.

The other type of post-translational control that has received attention is the modification of proteins subsequent to their synthesis by limited proteolysis. Sadoff and his co-workers have described the specific in vitro conversion by the sporulation serine protease of vegetative fructose-1,6-diphosphate aldolase into the spore enzyme (259). This proteolytic cleavage increased the thermal stability of the enzyme. The sporulation serine protease was also reported to release an antibiotic by proteolysis of some component, probably the ribosomes, of a vegetative cell extract (258, 318). The functional significance of this reaction is difficult to assess. The original observation that proteolytic modification of the β subunit of RNA polymerase occurred during sporulation has now been shown to be the result of proteolysis during extraction (186, 222) and therefore not of functional significance. Thus, at present, no evidence exists that limited post-translational proteolysis of proteins is involved in the regulation of sporulation, although it could well play a role in the preparation of spore components for dormancy.

CONCLUSIONS

As will be clear from the foregoing account, considerable doubt exists about the precise role that most sporulation-associated events play in the regulation of the process. Nonetheless, some general aspects with regard to control of the process, which have arisen for the most part from genetic studies, can be stated with more certainty and must be included in any model. It seems likely that sporulation is brought about by relief from repression by nitrogen-containing phosphorylated metabolite or metabolites; it is not clear whether there is one or more than one repressing compound. The weight of evidence is in favor of different mechanisms operating to overcome the repression of sporulation and to overcome the catabolite repression of inducible enzyme synthesis. This can be summarized as follows: stage 0 mutants that were hyper-repressed as regards all sporulation events were not impaired in their ability to synthesize inducible enzymes (37), and were found to grow normally on all substrates tested that required the synthesis of inducible enzymes for their metabolism (P. Schaeffer, personal communication). Mutants insensitive to glucose or NH_4^+ repression of sporulation were normal with respect to catabolite repression of inducible enzymes (154). Sporulation could only be initiated while DNA was being replicated, whereas the induction of enzyme synthesis was independent of DNA replication (51). Indeed, the analogy of spore formation with vegetative cell division is much stronger than any analogy with the synthesis of inducible enzymes. Nevertheless, it remains possible that there is some common factor involved in the repression of sporulation and the catabolite repression of inducible enzyme synthesis.

All *spo* mutants are pleiotropic, and this must indicate some sort of dependent sequence for the subsequent events. This aspect is best accommodated in a system of sequential induction (20, 118, 196, 289) in which expression of one locus (or set of loci) is responsible for activating the transcription of the next locus required in the sequence. This scheme can be refined to include the inactivation of transcription when the gene products are no longer required. The possible regulatory role of an altered template specificity for RNA polymerase, or for the machinery for protein synthesis, is essentially a ramification of the sequential induction model.

It is now clear that there are at least 30, and probably between 40 and 50, sporulation loci (143), which are widely dispersed on the chromosome. If it is assumed that they are only expressed during sporulation, then most, if not all, of them represent separate control points or operons (227). Although the location of many *spo* loci is known, the products of the loci are unknown and it is not yet possible to construct a coherent model for the expression of the loci. It is clear, however, that a single linear dependent sequence of induction does not represent an adequate model for the process. This is indicated (i) by the phenotype of some late-blocked

mutants which, for example, may be in one instance coat$^-$ cortex$^+$, and in another coat$^+$ cortex$^-$; and (ii) by the phenotype of certain constructed double mutants, which become blocked at an earlier stage than the parent strains possessing either of the single mutations (52). For example, there is strong evidence for saying that the *spoVC* locus and probably the *spoIVA* locus are not involved in the same sequence of events as the *spoIVF* locus. Third, the extensive studies of spore coat biosynthesis have indicated that this component is synthesized well before it is finally assembled (6a). These observations make it necessary to refine the scheme of sequential induction to include parallel pathways in a manner illustrated in an earlier section. The commitment experiments indicate that a number of sporulation loci must be sensitive to some form of catabolite repression as well as to induction by preceeding events in the process of spore formation. The results obtained with temperature-sensitive mutants indicate that other refinements may be required. These studies have shown that some sporulation gene products, such as that from the *spoIIG* locus, are required for only a short time during the process, whereas others, such as that from the *spoVB* locus, are required to be produced for a much longer time (335).

It remains convenient to make a distinction between vegetative functions necessary for sporulation (mutations in which would be expected to affect both growth and sporulation), and sporulation-specific functions (mutations in which would affect only sporulation). We have considered mainly loci presumed to be involved in spore-specific functions, but it is clear that only about 20% of the RNA species found in vegetative cells are replaced by new RNA species during sporulation (64, 226, 292), and many vegetative functions continue to be expressed during sporulation (166). Any future description may well have to account for the continued activity of many vegetative functions necessary for sporulation (as well as the inactivation of vegetative functions that are inhibitory).

Much of the genetic analysis of sporulation has followed methods developed for the analysis of metabolic pathways. However, some of these have not proved useful, and it is clear that a somewhat different approach is required for the genetic analysis of sporulation (and presumably other developmental processes); it is not merely that one system is a more complex version of the other. The first major difference is that all *spo* mutations are pleiotropic. The second is that the sequence of expression of *spo* loci is much more delicately controlled than sequences of induction of metabolic enzymes. In certain circumstances, induction of late genes of a metabolic sequence may occur without the induction of the earlier genes; there is no evidence for such a situation in spore formation. Mutants blocked in sporulation have been shown to lose the ability to form spores within, at most, a few hours of reaching the block (335). This behavior is quite unlike that of mutants blocked in metabolic systems, where is it often possible to supply the mutants with the missing intermediate at any time for them to complete their pathway. It would explain why no cross-feeding between sporulation mutants has been observed. As a consequence of these factors, no primary product of a *spo* locus has yet been identified, and in no case can the primary product be assayed quantitatively. This sets severe limitations on the interpretation of analyses of merodiploids. Thus it is not surprising that some of the "classic" methods of analysis have been vitiated. It is likely that these restrictions will also apply to the genetic analysis of other, more refractory, developmental systems, such as cell division, for which spore formation may provide a useful model.

APPENDIX

Characterization of the Sporulation Loci in *B. subtilis* 168

For description of the biochemical properties of the mutants, the following abbreviations are used: Pr, exoprotease activity; Ab, antibiotic production; AP, alkaline phosphatase activity; GDH, glucose dehydrogenase activity; DPA, formation of dipicolinic acid. To determine phenotypes, mutants were generally incubated in conditions that caused the wild type to sporulate, and their properties were compared with those of the wild type sporulating under the same conditions.

spo0 Loci

Originally stage 0 was used to describe mutants whose nuclear material resembled that of the growing bacterium as seen in electron micrographs of thin sections of fixed bacteria. Stage I represented appearance of the axial filament of nuclear material. Most workers have found difficulty in distinguishing unequivocally between these two phenotypes in *spo* mutants. It has recently been shown that this change in appearance of chromatin does not require protein, DNA, or RNA synthesis, and seems to be a physical reaction to the change in chemical environment (199). Thus, genetic expression is not required and separate mutations for this stage will presumably not occur.

For clarity, all mutations that cause sporulation to be blocked before formation of the spore septum are designated *spo0*.

Michel and Cami (203) subdivided stage 0 mutants into three phenotypic classes: 0A, which produced no exoprotease or antibiotic activity (Pr^-Ab^-), 0B ($Pr^\pm Ab^-$), and 0C (Pr^+Ab^+). This classification has been useful for ascribing a biochemical phenotype to *spo0* mutants, but it should not be confused with the locus designations. To avoid confusion we have not used 0A, 0B, 0C as the phenotypic designations.

spo0A

Various workers have located *spo0* mutations in the region of the chromosome between *phe* and *lys* (Fig. 2) and linked to *lys* by transduction (50, 137, 148, 152, 153, 227, 244, 306, 307). In transformation crosses many of these map in a small region of the chromosome probably representing only one or two genes (133, 143, 148, 213; J. A. Hoch, personal communication); this is designated *spo0A* (Table 1). Several *spo0A* mutations were suppressed by a presumed nonsense suppressor, indicating that the locus codes for at least one protein (137). Mutations mapping in this group can give rise to different levels of protease and antibiotic; Michel and Cami (213) reported that there were two types, $Pr^- Ab^-$ and $Pr^+ Ab^+$, which were called sp_{0A} and sp_{0C}, respectively. Mutations of the $Pr^+ Ab^+$ type mapped close to, or in, the same region as $Pr^- Ab^-$ type in transformation crosses (213), and for this reason both types are considered to be in the *spo0A* locus, which is therefore clearly complex. In studies where mutants were not classified by morphological stage of blockage, only the $Pr^- Ab^-$ phenotype was associated with the *spo0A* locus, as the $Pr^+ Ab^+$ type could not have been distinguished from later-blocked mutants (137, 244). The requisite transformation crosses for several mutations mapping in the region have not been performed. These mutations are assigned tentatively to the *spo0A* locus, and are designated *spo0A*^{c} in Table 1.

Mutations in the *spo0A* locus and of the $Pr^- Ab^-$ type cause the most pleiotropically negative phenotype of all sporulation mutants (37; Table 2). As already discussed, they do not form protease or antibiotic. Nor do they show any of the biochemical or morphological changes associated with sporulation. Thus, they are often poorly competent (286); they are permissive hosts for bacteriophage $\phi 2$, $\phi 15$ (152), and ϕe (40); they are sensitive to *B. subtilis* antibiotic and to polymyxin (112, 151); they are altered in regulation of cytochrome (303) and nitrate reductase formation (33, 114). In addition, they show none of the changes associated with later stages: formation of alkaline phosphatase, glucose dehydrogenase, or dipicolinate (49, 227, 320). They are also distinguishable from the wild type in that they produce large quantities of an insoluble, semicrystalline protein (286), the significance of which is not known.

spo0G

Ionesco et al. have described a mutation, 14 UL, that is cotransduced with *lys* and causes a $Pr^- Ab^-$ phenotype, but is not linked to *spo0A* in transformatin crosses (148). Thus, 14 UL lies in a separate locus designated *spo0G*. The strain showed wild-type competence to be transformed and so had a later phenotype than *spo0A* mutants (148).

spo0D

Ionesco et al. reported a $Pr^+ Ab^+$ stage 0 mutation that was weakly linked to *phe-1* by transduction, and also linked to the *acf* marker that lies between *phe* and *lys*. It was not linked to any of the adjacent *spo0* loci (148) and so lies in a separate locus.

spo0B

Ionesco et al. (148) and Hoch and Mathews (136) have both reported a group of $Pr^\pm Ab^-$ stage 0 mutations that were both cotransducible and cotransformable with *pheA1*. The two groups of mutations are closely linked to each other in transformation crosses (J. Hoch, personal communication) and so they are placed in a single locus. The two reports differ in their suggested orientation of the locus relative to outside markers. We favor the order *leu spo0B pheA* (136). Hoch and Matthews used the linkage to *phe-1* to construct a fine-structure map of the locus by three-factor transformation crosses; the order obtained was not that expected from the degree of linkage to *phe-1*. The recombination indexes suggested that the locus is composed of one or at the most two genes (136).

Phenotypically, *spo0B* mutants rank after *spo0A* in the completeness of their loss of sporulation-associated properties (Table 2).

spo0F

Hoch and Mathews described a group of mutations that fell into a tight cluster in transformation crosses, but could not be linked to any markers on the genetic map as it was known at that time (136). These have since been shown to be cotransformable with *ctrA1*, confirming that they lie in a "new" part of the map (J. Hoch, personal communication). An anomaly arises

in that these mutations are cotransformable (J. Hoch, personal communication) with another group (*spoB loc-1*) of $Pr^{\pm}$ Ab^{-} mutations (113, 203) of which one, 3U, had been shown to be cotransducible with the distant *acf* marker (148). This discrepancy remains to be resolved; for the present, we have favored the transformation over the transduction data.

spo0F mutants show a later block than *spo0B*, being normal in their cytochrome phenotype (303) and "variable" in their ability to allow ϕ15 to form plaques (37).

spo0H, spo0J

A number of *spo0* mutations have been linked by transduction to *cysA14* or other markers in that region (136, 143, 227, 307), and represent at least two loci (143). Hoch (personal communication) has confirmed by transformation crosses between *spo* mutants that many of these should be considered to lie in a single locus designated *spo0H* (136). Several of the mutations were cotransformable with *cysA14* and with *rif* (227). Some attempts to order mutations in this region by transformation crosses have been reported to give ambiguous results (104, 105, 136). This may be because strains that were originally considered to have a single (*cysA14*) mutation have been shown to have linked auxotrophic mutations *cys* and *cym* (requiring cysteine or methionine) (156, 227, 228). Nevertheless, Piggot (227) deduced the order *cysA spo0H rif* from three-factor transformation crosses.

Two *spo0* mutations studied by Hranueli et al. (143) were loosely linked to *cysA14* by transduction (48 and 49% cotransduction) and were not linked to this marker by transformation. They were linked to each other by transformation and not to *spo0H* mutations. Thus, they define a further locus, *spo0J*. Hoch (personal communication) has established the marker order *cysA ery spo0J*. For various other mutations linked to *cysA14* by transduction (Table 1) no transformation crosses have been reported. These are tentatively assigned to *spo0H*; in several cases, the cotransduction frequency with *cysA14* make it unlikely that they could lie in *spo0J*.

Phenotypically the *spo0H* mutations are less pleiotropic than mutations in the other *spo0* loci discussed so far (Table 2). Brehm et al. (37) reported that *spo0H* mutants were normal in their production of protease and antibiotic, whereas Piggot (227) described other mutants that produced little protease and no detectable antibiotic. One of the later mutants, E22, has been characterized extensively (201) and also shows reduced protein turnover. These variations may reflect genuine differences between mutants, or that may be a consequence of the differences in the methods for inducing sporulation or of protease assay between the two laboratories.

The *spo0J* mutants have not been extensively characterized: they produced antibiotic and protease (P. J. Piggot, unpublished observations).

spo0K

A single *spo0* mutation has been located between *metC3* and *argC4* (50); it is cotransformable with *trpS1* (J. A. Hoch, personal communication). The locus is designated *spo0K*. Under sporulating conditions, cells of the mutant divide to give short, almost square cells, which have a Pr^{+} Ab^{+} phenotype (49).

spo0E

A series of mutations have been linked to *ura* (50, 136, 295). As all lead to an oligosporogenous phenotype, no transformation crosses have been performed between the different strains. The variation in transduction linkage (Table 1) suggests that there may be more than one locus. However, the fluctuations in cotransduction frequencies, together with the known anomalies in the region (229), have prompted us to include only one locus, *spo0E*.

The mutants described by Coote (49) formed approximately 20% heat-resistant spores, making biochemical characterization difficult. The mutants described by Brehm et al. sporulated to a lesser extent, and were $Pr^{\pm}$ Ab^{-} (37). Bacteriophage ϕ15 and ϕ2 had low, but significant, plating efficiences on these mutants.

spoII loci

Strains with *spoII* mutations are able to form the spore septum. Their development may proceed further than this in normal or aberrant ways, but they are unable to form a detached prespore within the mother cell. Several phenotypes associated with this stage have been described (49, 238, 256, 257, 320, 329, 333). Ryter et al. (257) distinguished between mutants where a single septum was made at one pole of the cell and mutants where septum formation occurred at both poles of the cell. Considerable cell wall material is often visible in the septa (256, 333). With both phenotypes, the larger compartment often lyses, leaving the smaller "pygmy" cell intact; this behavior can be observed with a phase-contrast microscope. Multiple septate mutants have also been described (238, 256, 320, 329). However, different cells within a single population of certain mutants may have one, two, or multiple septa (227, 329),

so that this phenotypic distinction may be misleading in distinguishing loci. Biochemically, stage II mutants are always Pr^+ Ab^+, and indeed often overproduce protease (B. N. Dancer, D. Phil. thesis, University of Oxford, 1974). Properties that distinguish mutations from the different loci are shown in Table 3.

spoIIA

Ionesco and Schaeffer (149) reported five stage II mutations that were cotransformable with *lys-1*; these were also linked to each other in transformation crosses (148) and define the *spoIIA* locus. One of the mutants was reported to form a single septum, whereas the other four were abortively disporic (these phenotypes were designated type A and type B, respectively, but this designation is not used here). The mutation causing a single septum showed no recombination with two mutations causing two septa (250), and Rouyard et al. concluded that allelic mutations could differ in this respect. The mutations were closely linked to *lys-1* by transduction (148). Mutations described by Coote (50) and Piggot (227) were also strongly cotransduced with *lys-1*, and in all cases the order obtained by three-factor cross was *spo lys trp*, so that these almost certainly lie in the same locus. The oligosporogenous mutant described by Coote (49) was abortively disporic (Fig. 3), whereas the six asporogenous mutations mapped by Piggot could give rise to one, two, or multiple septa (227; D. A. Wood, personal communication). Takahashi described a stage II mutation linked to *ser* (306, 307), which is in the same region of the chromosome; the assignation of this *spo* mutation to *spoIIA* is more tentative. Biochemically, the mutants examined by Coote (49) and Piggot (227) were AP^-.

spoIIB

Coote (50) located an oligosporogenous mutation linked to *phe-12* and *leu-8*, but its orientation with respect to these markers was not determined. The mutant formed a single septum, which was straight and did not bulge into the mother cell; the septum contained wall material. The mutant was AP^+.

spoIIC

Coote (50) located on oligosporogenous mutation linked to *cysB3* and *hisA1* (58 and 43% cotransduction, respectively). As these markers are only 20 to 24% cotransduced (70), the *spo* mutation was placed between them. The mutant, P9, formed a single spore septum in the normal way, but the septum membrane bulged into the mother cell in the middle while remaining attached to the original cell wall invaginations or spikes (Fig. 4). Presumably, the mutant was unable to digest away the peptidoglycan synthesized at this time. Biochemically, the mutant was AP^+.

spoIID

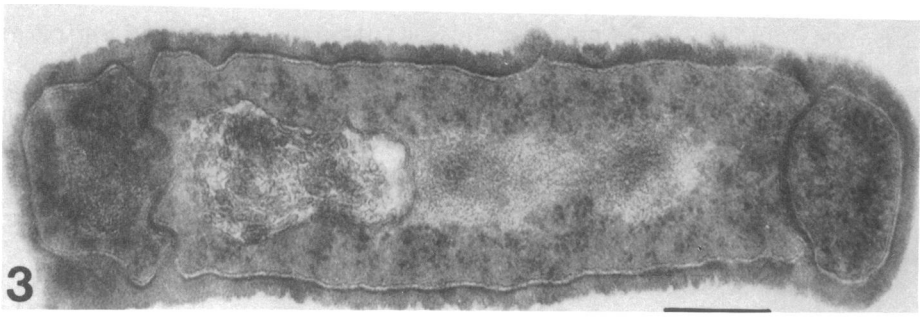
Piggot reported two other stage II mutations that were cotransducible with *hisA1* (227). These were not linked to *cysB3* and have since been found to be cotransducible with *ctrA1* (P. J. Piggot, unpublished observations), and so presumably define a distinct locus that is tentatively placed between *hisA1* and *ctrA1*. The mutants formed a single septum that did not bulge into the mother cell (52); they were AP^+.

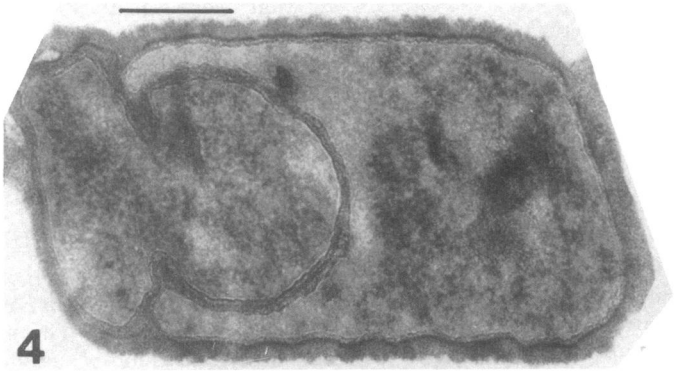
spoIIE

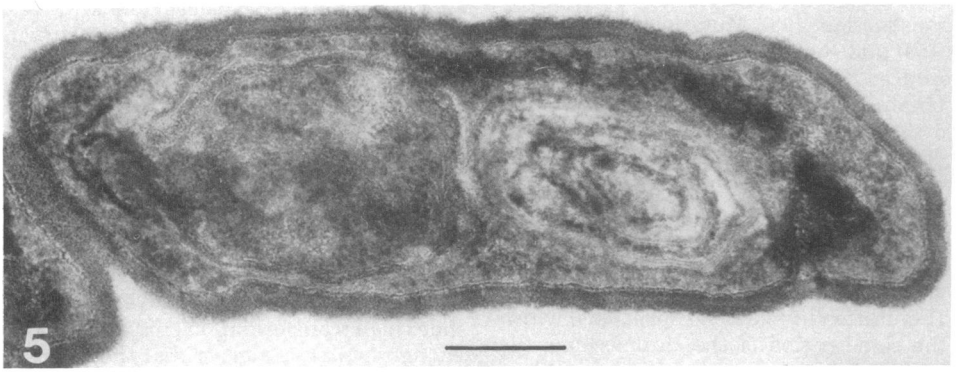
There are several reports of stage II mutations mapping in the *purA cysA ery* region (50, 143, 227, 306, 307). The most fully studied of these is a group of eight described by Piggot (227). All were cotransformable with *cysA14*. The mutations gave rise to three distinct phenotypes, all of which were AP^-. (i) One mutant (N25, see [320]) produced excess membrane within the cell; this suggested that development was blocked just before formation of a free protoplast (stage III). However, epistasis experiments indicated that it was blocked early in stage II (52), and this was consistent with the AP^- phenotype. (ii) Five mutants had the abortively disporic phenotype. Their mutations, together with that of N25, gave recombination indexes in transformation crosses that were low enough (<0.1) to suggest that they were allelic (227). Hranueli et al. have since added a mutation to this group (143). (iii) Two mutants laid down several septa that were rigidified by thick cell wall deposition (D. A. Wood, personal communication). Their mutations gave slightly higher recombination indexes (generally 0.05 to 0.3) in transformation crosses with the other mutations (227). This suggests a small cluster of genes, but all the mutations are placed in a single locus, *spoIIE*. Three-factor transformation crosses were consistent with the order *spo cysA rif* (227). The other stage II mutations that have been mapped in this region by transduction (50, 306, 307, Table 1) are tentatively placed in the same locus.

spoIIF

Hranueli et al. (143) reported a *spoII* mutation linked to *metC3* and *ura-1* (49 and 21% cotransduction, respectively). The mutant was abortively disporic and AP^-. Transformation crosses indicated that this mutation was un-







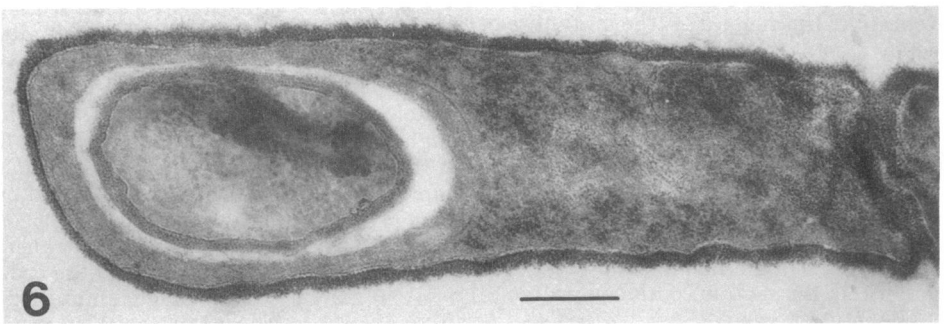


FIG. 3. *Mutant P18 (spoIIA) has formed spore septa at both poles of the cell and has laid down cell wall material between the septum membranes. Bar represents 0.2 μm in this and subsequent micrographs.*

FIG. 4. *Mutant P9 (spoIIC) has formed a single septum which has grown into the mother cell in the normal way, although it has remained attached to the original cell wall invaginations.*

FIG. 5. *Mutant P20 (spoIVA) has developed normally up to the free prespore stage, but the coat material is laid down in the mother cell cytoplasm instead of around the prespore.*

FIG. 6. *Mutant P7 (spoIVB) shows almost complete cortex development, but coat formation has been arrested at an early stage. Taken from Coote (49) with permission of the publisher.*

linked to mutations in the *spoIIG* locus (143, 334). Two abortively disporic mutations described by Ionesco et al. (148) are tentatively placed in *spoIIF* (Table 1) rather than *spoIIG*, because of their low frequency of cotransduction with *ura*.

spoIIG

Piggot located three *spoII* mutations in this region (227); two of these were closely linked to *ura-1* (83% cotransduction). A fourth mutation, NG4-14, originally described as having a stage 0 phenotype, has since been shown to cause a block at stage II (D. Hranueli, personal communication). The four mutations were initially thought to lie in two transformation groups (227). However, a more comprehensive analysis has shown that they map in a single transformation group, together with mutation 279, which gives rise to a temperature-sensitive stage II phenotype (334). They thus define a single locus, *spoIIG*. In a series of three- and four-factor crosses, Young established the map order: *metC3 spoIIF spoVE spoIIG furA2 cysC7 spoIIE* (334). Mutation *spoIIG* 279 was 46% cotransformed with mutation *spoVE85* (334). The *spoIIG* mutants had the abortively disporic, AP^-, phenotype.

spoIII loci

Mutation in any of these loci does not prevent formation of the spore protoplast, but does prevent formation of the germ cell wall and cortex layers. The mutants are invariable Pr^+ Ab^+ AP^+ DPA^-. However, they are reported to differ in their ability to form glucose dehydrogenase (49, 320).

spoIIIA, spoIIIB

Piggot (227) located seven stage III mutations linked to *lys-1* (43 to 56% cotransduction). Six of these were linked to each other by transformation and are placed in the *spoIIIA* locus; the recombination index across the group of 0.51 suggested more than one gene (227). A tentative map of the locus was constructed from the recombination indexes. The seventh mutation is placed in the *spoIIIB* locus. Three mutations characterized by Hranueli et al. (143) were linked to the first group by transformation. Ionesco et al. (148) and Coote (50) had also located stage III mutations in the region of *spoIIIA* and *spoIIIB;* these are all tentatively placed in the *spoIIIA* locus (Table 1), pending the requisite transformation crosses. Three-factor transduction crosses were consistent with the order *spo lys trpC2* for both loci (227). Mutations in this group give rise to three distinct morphologies (320; D. A. Wood, personal communication): (i) cells reached stage III normally and development stopped; (ii) the spore protoplast was formed normally, but continued incubation caused the mother cell to lyse, leaving a cell envelope containing various membranous elements; (iii) the spore protoplast was formed normally, but the mother cell retained the wall invaginations where a spore septum had presumably been laid down. Biochemically, some of the mutants were GDH^+ and others GDH^- (49, 320); the negative results obtained by one of us (227) may reflect the difficulties of assaying the enzyme in mutants. Both phenotypically and genetically there would appear to be several closely linked genes concerned with stage III.

spoIIIC

This is defined by mutation 94U which is weakly linked to *phe-1* and *ilvC1* and not linked to *lys-1* by transduction (148). However, the mutation in question may correspond to mutation Sp^-III94, whose phenotype was described by Ryter et al. (257). If this is the case, then the phenotype suggests that the mutation should not be included in a stage III locus: the mutant cells of 94 were able to synthesize a germ cell wall, and continued incubation caused lysis of the mother cell and eventual release of unfinished, but intact, prespores into the medium. This phenotype is associated with *spoIVC* and *spoIVD* mutations that map in the same region (up to 25% cotransduction with *phe-12*, see below). For this reason, the existence of a separate *spoIIIC* locus is open to doubt.

spoIIID

A stage III mutation was linked to *hisA1* by transduction (148). A second mutation had been shown to be closely linked to the first in transformation crosses (250). No phenotypic description of the mutants has been published.

spoIIIE

Three stage III mutations have been described that are cotransduced with *ura-1* (36 to 47%) and are linked to each other in transformation crosses (143, 227, 334). They were very weakly cotransduced with *metC3* (about 1%), and multiple factor crosses suggested the order *metC3 furA2 ura-1 cysC7 spoIIIE* (334). It should be noted that this data indicated a revised order for the *furA2, ura-1,* and *cysC7* markers. A stage III mutation that had been weakly linked to *ura-26* (12% cotransduction) by Ionesco et al. (148) is conservatively placed in the same locus, as is the mutation that had been located in the region by Coote (50). In the latter case, the rather high linkage to *metC3*

(49% cotransduction) suggests that this might lie in a different locus, but this has not been established. The mutant described by Coote reached stage III normally, and then development stopped; it was GDH^- (49).

spoIV loci

Mutations are included here that allow germ cell wall and, in some instances, cortex synthesis between the double membranes of the protoplast, but do not allow deposition of spore coat around the prespore. We have included mutations giving rise to an unusual aberrant phenotype in this group (*spoIVA*). The mutants in *spoIV* loci may be distinguishable by these morphological criteria, but they are not readily distinguishable by biochemical criteria. All the mutants that have been tested are Pr^+ Ab^+ AP^+ GDH^+. The prespores achieve a low degree of refractility (phase-grey appearance), which is almost diagnostic for stage IV to V mutants; prolate prespores ("sporlets") are often released into the medium. No stage IV mutant has been found to synthesize DPA, and they do not show resistance to organic solvents, such as octanol.

spoIVA

Coote (49) described an abnormal oligosporogenous mutant in which the spore developed normally up to the synthesis of the primordial germ cell wall. Little cortex was made, and coatlike material was deposited in concentric layers in the cytoplasm of the mother cell but not around the spore protoplast (Fig. 5). When viewed with a phase-contrast microscope, each cell appeared to contain two phase-grey bodies. The mutation mapped between *lys-1* and *trpC2* (50, Table 1). A completely asporogenous mutant having an identical phenotype has also been described (227). A recombination index of 0.07 was obtained in a transformation cross between the two strains, suggesting that the two mutations are located in the same gene (P. J. Piggot, unpublished observation). The *spo* mutation was cotransformable with *trpC2* (227).

spoIVB

A single stage IV mutation has been linked to *lys-1* and *trpC2*, and placed in the order *spoIVB-lys-1-trpC2* by three-factor transduction cross (49, 50). The cells of this oligosporogenous mutant that were not making complete spores developed an almost complete cortex, but little or no coat deposition occurred (Fig. 6).

spoIVC

Coote (49, 50) found five stage IV mutations that were weakly linked to both *phe-12* and *lys-1* (9 to 25% and 1 to 4% cotransduction, respectively). A maximum recombination index of 0.24 was obtained in transformation crosses between the mutants, suggesting that the mutations lie in a locus that might have more than one gene. All the mutants had a similar phenotype. The cells formed the primordial germ cell wall, seen as a dark-staining area between the double membranes of the prespore (Fig. 7), but no light-staining cortex. Prolonged incubation caused the mother cells to lyse and release stable unfinished prespores into the medium; these "sporlets" were mechanically intact, but there is no evidence that they were viable. Two of the mutants were oligosporogenous in character, where the remaining three were asporogenous. Transformation crosses between one oligosporogenous and two asporogenous mutants gave recombination indexes low enough (<0.1) to suggest that they were located in the same gene (50). A mutant with a similar phenotype was studied by Hranueli et al. (143), and transformation crosses indicated that the mutation was located in the same locus.

spoIVD

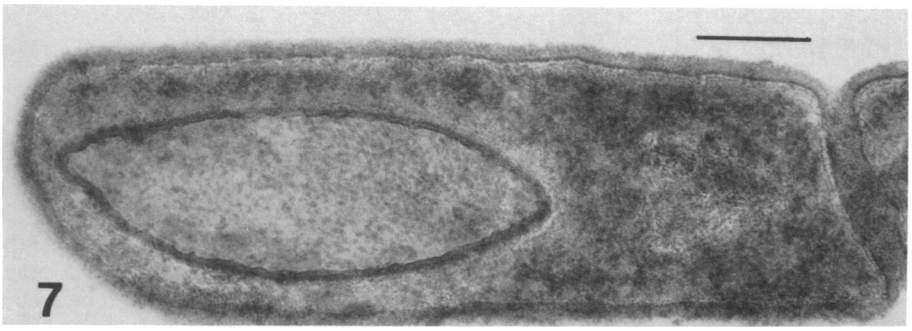
This locus is defined by a mutation linked to *phe-12* and *leu-8* (30% and 12% cotransduction respectively) by Hranueli et al. (143). Three-factor transduction crosses were consistent with the order *leu-8 phe-12 spoIVD*. Transformation crosses showed that it was distinct from the *spoIVC* locus, which is in the same region and is associated with a similar phenotype. As *spoIVC* mutations were not cotransduced with *leu-8*, and the *spoIVD* mutation was not cotransduced with *lys-1*, the order *leu-8 phe-12 spoIVD spoIVC lys-1* is suggested.

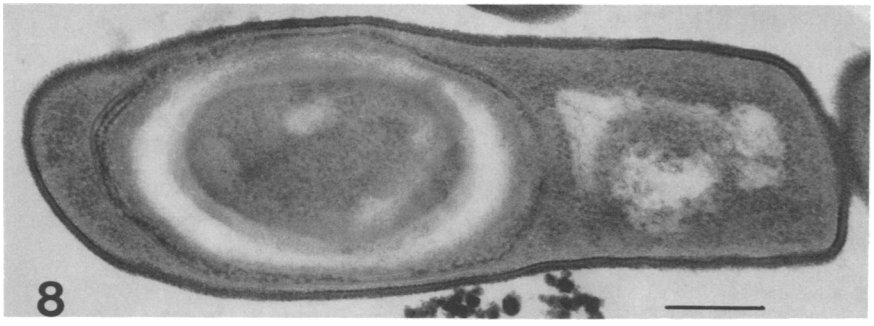
spoIVE

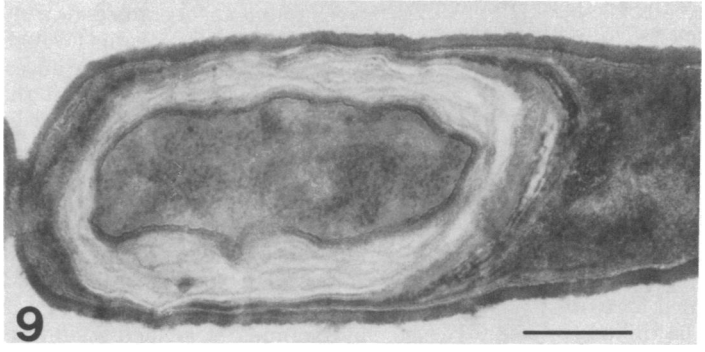
Ionesco et al. (148) have described a stage IV mutation that also maps in the region of *spoIVC* and *spoIVD* (47% cotransduced with *phe-1* and 3% with with *lys-1*). This mutation gave rise to a distinct phenotype in that the mutants formed an almost complete cortex (213). For this reason the mutation is placed in a separate locus, although no transformation crosses have been performed with *spoIVC* and *spoIVD* mutants. From the published data it is not possible to order *spoIVE* relative to *spoIVC* and *spoIVD*.

spoIVF

A group of three oligosporogenous mutations were located close to *phe-12* (89 to 97% cotransduction) by Coote (49, 50). A mutation studied by Hranueli et al. (143) gave recombination indexes with the three oligosporogenous muta-







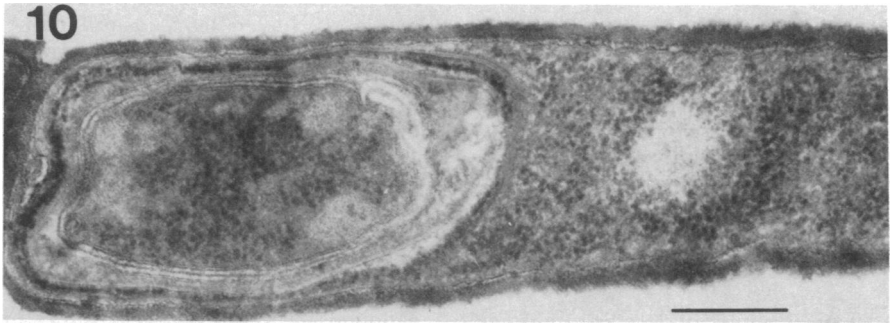


FIG. 7. *Mutant Z28 (spoIVC) has developed as far as the first stage of cortex formation, the primordial germ cell wall. This is seen as an electron-dense band between the prespore membranes. Taken from Coote (49) with permission of the publisher.*

FIG. 8. *Mutant 89 (spoVA) has developed almost complete cortex and coat layers, but much detail of the spore core remains visible. Micrograph courtesy of D. Hranueli.*

FIG. 9. *Mutant W10 (spoVD) has formed a cortex with an unusual striated appearance and development of the coat layers is only complete at the poles of the spore. Taken from Coote (49) with permission of the publisher.*

FIG. 10. *Mutant W5 (spoVE) shows well-developed coat layers in the absence of normal cortex formation. Taken from Coote and Mandelstam (52) with permission of the publisher.*

tions that were low enough to indicate that the four mutations lay in a single locus. Three-factor transduction crosses indicated the order *leu-8 spoIVF phe-12* (143). This is a different position from that originally suggested, on the basis of two-factor transduction crosses (50). The mutants had the same phenotype as *spoIVC* mutants.

spoIVG

A stage IV mutation was linked to *arqC4* (47% cotransduction) by Piggot (227). A second mutation showed no recombination with this in transformation crosses, and so both are placed in the same locus. In the light of recent alterations to the *B. subtilis* genetic map (182) the reported weak (1% cotransduction) linkage with *hisA1* must be regarded as spurious. The mutants made an almost complete cortex (320).

spoV loci

Mutations that allow deposition of coat around some or all of the prespore are included in this category. Not all allow normal cortex synthesis. Biochemically, the mutants were all Pr^+ Ab^+ AP^+ GDH^+. They could often be distinguished by their ability to synthesize DPA and to develop resistance to organic solvents, such as octanol and chloroform. In most instances the loci have been identified by a single mutation.

spoVA

This locus is defined by a stage V mutation that was shown to be linked to *lys-1* (61% cotransduction) by Hranueli et al. (143). Orientation of the locus relative to *lys* was not determined. The authors could not establish linkage by transformation to any of the stage IV loci in the region. The mutant was octanol sensitive and produced DPA (P. J. Piggot, unpublished observations). Morphologically, the cortex appeared normal and the prespores were surrounded by coat material (D. Hranueli, personal communication; Fig. 8).

spoVB

This is defined by mutant 91 of Hranueli et al. (143). The mutation was closely linked to *phe-12* (18% cotransformation), and three-factor transduction crosses gave the order *leu-8 phe-12 spoVB*. The mutant was originally listed as stage IV (143); however, it formed coat material around part of the prespore (D. Hranueli, personal communication) and so is reclassified as stage V. Although coat material is seen, cortex formation was incomplete. This mutant produced DPA (P. J. Piggot, unpublished observation).

spoVC

Coote (49, 50) located an oligosporogenous stage V mutation linked to *cysA14* (70% cotransduction). Most cells of the mutant made a complete cortex and normal coat layers, but the normal decrease in electron density of the spore core during maturation did not occur, and much detail of the protoplast remained visible. This suggested that the maturation process was incomplete in this strain and this was borne out by the absence of DPA and the inability of the developing spore to achieve octanol resistance. A temperature-sensitive mutant classed as stage IV to V, with a mutation linked to *cysA14* (79% cotransduction) has recently been reported by Young (335). This is tentatively placed in the same locus. The orientation, with respect to other markers on the chromosome, is not known for either mutation.

spoVD, spoVE

Two oligosporogenous stage V mutations were found to be linked to *ura-1* and *metC3* (50). In one of the strains (W10) the cortex had an unusual striated appearance, and the coat layers were complete only at the poles of the spore (Fig. 9). The mutant produced a normal amount of DPA, But 50% of it was lost to the surrounding medium (49). In the other strain (W5), the prespores were surrounded by well-developed coat layers, but the cortex was almost entirely absent (Fig. 10) and no DPA synthesis occurred. The "spores" of both mutants were sensitive to octanol. A mutant, 85, studied by Hranueli et al. had a similar phenotype to W5 (143). The mutations in these two strains were closely linked by transformation (RI = 0.08), and were weakly linked by transformation (RI = 0.4 to 0.6) to the mutation in strain W10 (143). Thus, there are good grounds for having two loci: *spoVD* (W10) and *spoVE* (W5, 85) in the region. Strains with similar phenotypes have been described by Balassa and Yamamoto (23, 24), and by Millet and Ryter (213), but the mutations have not been mapped.

spoVF

An oligosporogenous stage V mutation was found to be unlinked to the auxotrophic markers that were then available (49, 50), and so is genetically distinct from *spoVA-E*. Consequently, it represents an additional locus. The mutant developed apparently normal cortex and coat layers, but full maturation of the spore did not occur. The cells achieved resistance to octanol, but remained heat sensitive and did not synthesize DPA (49). This again distinguishes it from *spoVA-E* mutants. A mutant with similar phenotype has been described by

Balassa and Yamamoto (24); in this case, mapping was not attempted.

spo-5NG

Rogolsky (244) described an asporogenous mutation, 5NG (since designated *spo-68*, see 69, 331), which was closely linked to *metB10* (88% cotransduction) and weakly linked (27% cotransduction) to the *lys* marker of strain GSY 254 (this marker was listed as *lys-2* by Rogolsky, but has been listed as *lys-1* elsewhere, 148). It is clearly distinct from any of the mutations described previously and so identifies an additional locus. The mutant was only classified as Pr^+ Ab^+, and so could be blocked at any of the stages of sporulation.

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